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REDACTORES:

E. MUSTAKALLIO (TURKU) U. UOTILA (HELSINKI) ARMAS VARTIAINEN (HELSINKI)

A. WILSKA (HELSINKI) A. I. VIRTANEN (HELSINKI)

EDITOR

K. O. RENKONEN

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DEGRADATION OF RIBONUCLEIC ACID BY FUNGUS EXTRACTS

BY

REINO MÄKITALO

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MERCATORIN KIRJAPAINO
HELSINKI, FINLAND

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Reino Mäkitalo

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REINO MÄKITALO

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Helsinki, August 1959

R. M.

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ABBREVIATIONS

DNA	=	deoxyribonucleic acid
DNase	=	deoxyribonuclease
RNA	=	ribonucleic acid
RNA-P	=	ribonucleic acid phosphorus
RNA-P^{32}	=	ribonucleic acid labeled with P^{32}
PRNase	=	crystalline pancreatic ribonuclease
TLRNase	=	tobacco leaf ribonuclease
TMV	=	tobacco mosaic virus
TMV-RNA	=	tobacco mosaic virus ribonucleic acid
KU	=	Kunitz Unit, unit of ribonuclease activity
mc.	=	millicurie
AMP	=	adenosine-5'-monophosphate
ADP	=	adenosine-5'-diphosphate
ATP	=	adenosine-5'-triphosphate
GMP	=	guanosine-5'-monophosphate
GDP	=	guanosine-5'-diphosphate
GTP	=	guanosine-5'-triphosphate
IMP	=	inosine-5'-monophosphate
IDP	=	inosine-5'-diphosphate
ITP	=	inosine-5'-triphosphate
CMP	=	cytidine-5'-monophosphate
CDP	=	cytidine-5'-diphosphate
CTP	=	cytidine-5'-triphosphate
UMP	=	uridine-5'-monophosphate
UDP	=	uridine-5'-diphosphate
UTP	=	uridine-5'-triphosphate

INTRODUCTION

It is well known that two main groups of nucleic acids are to be found in the living cell, the ribonucleic acid (RNA) group and the deoxyribonucleic acid (DNA) group. The latter occurs almost exclusively in the cell nucleus where, in combination with nuclear proteins, it forms the deoxyribonucleoprotein known to histologists as chromatin. The ribonucleic acids are found only in small amounts in the nucleus, chiefly in the nucleolus and chromosomes. However, they abound in the cell cytoplasm where, as ribonucleoproteins, they form the chief basophilic constituents of the cytoplasmic large granules or mitochondria, of the cytoplasmic small granules or microsomes, and of the non sedimentable cytoplasmic substance or cell sap. It is usually believed that RNA is mainly localized in the nucleoli and the microsomes of the cell.

Unfortunately we know less about the precise chemical composition of RNA than that of DNA. The reason for this relative ignorance is that the isolation of RNA in pure native form is very difficult to achieve. The study of isolated RNA preparations has shown that the essential element of the RNA structure, as for DNA, is the nucleotide. The sugar component is D-ribose, phosphorylated and combined in glycosidic linkage with the purines, adenine and guanine, and the pyrimidines, cytosine and uracil. Adjacent nucleotides are joined together by phosphodiester linkages which most probably involve the carbon atoms in the 3' and 5' positions of the ribose. There is also a possibility of branching of the polyribonucleotide chain through the formation of additional ester linkages at the 2' position of the sugar. The ribonucleic acids prepared from yeast and from mammalian tissues under reasonably mild conditions have high molecular weights, but they are not as highly polymerised as the corresponding DNA preparations. The molecular weights of different ribonucleic acids must be regarded as approximations, but values as great as 300,000 have been recorded. RNA, in

many respects, is a more labile molecule than DNA, and enzymatic digestion of RNA by the intracellular ribonuclease is often difficult to prevent completely during the isolation procedure. Methods for the isolation of native RNA, are however, steadily improving. The more they improve, the more similar to DNA are the products obtained. In the absence of absolutely reliable isolation methods, it is impossible to say whether many different molecular species of RNA are present in various cells. We do not know whether the many different fractions separated really pre-exist in the living cell or whether they are artificial breakdown products of a single native RNA. It is clear, however, that there must be many different forms of RNA, but there is no reason to believe that they are not all built up in the same way by the condensation of large numbers of nucleotides to form a polynucleotide chain, probably with frequent branches. The nature of the internucleotide linkages involved in the formation of the polynucleotides has been worked out chiefly for yeast RNA, which is most readily obtainable in bulk, and largely with the aid of enzymes, of which the most important is ribonuclease (PRNase). Within the limits of the present state of knowledge, however, evidence will be presented to show that there are marked differences in the chemical composition (bases ratio) of the RNAs present in different parts of the cell. Nuclear RNA is always significantly different in composition from cytoplasmic RNA. This is an important point because the possible nuclear origin of cytoplasmic RNA is a current topic of very lively discussion. Although the problem of heterogenecity complicates the chemical study of RNA structure, it is of considerable biological interest because it suggests that differences in RNA structure reflect differences in function.

Many extensive experimental works leave no doubt that nuclear RNA is a very labile substance, much more so than cytoplasmic RNA. Futhermore, RNA is obviously a more labile nuclear constituent than DNA. The marked lability of nuclear RNA, compared with DNA, has been confirmed by metabolic studies with isotopes. While DNA is a relatively stable substance, RNA usually has a high rate of turnover. Whether the high turnover rate of nuclear RNA means that nuclear RNA

is a precursor of cytoplasmic RNA is a question that must be shelved here. Although nuclear RNA displays unusual metabolic activity, it also shows great quantitative variations. The amount of RNA per cell nucleus not only varies from species to species, it can also vary in different organs of the same species. This latter finding is in sharp contrast to the constancy ascribed to the nuclear DNA content. It has also been found that the RNA content per nucleus may vary with changes in the physiological state of a tissue. Prolonged fasting, for example, results in a decrease in the ribonucleic acid content of liver nuclei. This variability is again in contrast with DNA stability both in amount and metabolism under the same conditions.

It can be concluded that the biochemical evidence and the cytochemical evidence stand in good agreement. Despite a few limitations, two interesting and important conclusions can be drawn from the results. First, the composition of the total nuclear RNA varies markedly from the average composition of the cytoplasmic RNA. Second, the nuclear ribonucleic acids vary in composition and amount from tissue to tissue. Thus, both the amount and the composition of nuclear RNA can vary in different tissues of the same organism, and the amount of nuclear RNA can vary in the same tissue under different conditions. The significance of such variations is not known at present, but they suggest that the ribonucleic acid of the nucleus is connected with metabolic activities in the rest of the cell. Tracer experiments using a variety of isotopically-labeled precursors have made it clear that the RNA of the nucleus has a unique metabolic activity. The high activity of nuclear RNA has prompted the suggestion that the nucleus is one of the main sites of ribonucleic acid synthesis for the cell as whole and, finally, there is considerable evidence which relates the ribonucleic acids to the process of protein synthesis. This may be said even if the bulk of the protein synthesis occurring in a cell is now known to be associated with the cytoplasmic ribonucleoprotein.

Experiments in recent years demonstrate a new and important aspect of the possibility of inhibiting growth and protein synthesis in living cells or organisms by an appropriate ribonuclease treatment. Very little, however, is known about the action of

ribonuclease in any tissue *in vivo*. The little that is known, as Brachet (14) pointed out, shows that ribonuclease can penetrate a number of cells and organisms. The enzyme does not interfere with the energy-producing mechanisms and it has complex effects on RNA metabolism. The enzyme-substrate complex formation is usually followed by enzymatic breakdown of the RNA. In all cases studied so far, incorporation of amino acids into proteins, protein synthesis, mitotic activity and growth have been drastically inhibited. The experiments strongly indicate that in living cells RNA integrity is essential for protein synthesis.

Recent advances in virology reflect the fundamental progress in cytochemical and biochemical analysis at the level of cellular phenomena. The first and major advance was the establishment of the primacy of nucleic acids in viral infection. Only RNA was found in the viruses affecting higher plants, whereas both RNA and DNA, either single or jointly, have been reported in highly purified preparations of animal viruses. Analysis of plant virus nucleic acids soon established that these samples of RNA had different and distinctive compositions, some being similar to and others differing radically from the classic prototype of RNA, yeast nucleic acid. Conclusions about the infectivity of plant virus RNA must be based largely on the tobacco mosaic virus (TMV), since relatively little is known about this aspect of other plant viruses. As shown above, intracellular RNA is regarded as a very reactive compound and its sensitivity for ribonuclease action has been reported in certain instances. In virus, however, RNA is protected against the ribonuclease by its protein shell, which has given it a reputation of chemical inertness. For example, TMV forms compounds with ribonuclease, but no digestion occurs and the virus enzyme complex can be split and intact viral nucleoprotein which is fully effective can be recovered. It has long been known that ribonuclease interferes actively in virus multiplication. The idea propounded later that only nucleoprotein, native or reconstituted, is infectious received its death-blow in 1956 with the different opinions advanced independently by Gierer and Schramm in Tübingen (35) and Fraenkel-Conrat in Berkeley (32). According to them, the pure ribonucleic acid itself,

isolated from TMV, rather than residual intact virus particles or residual parts of protein shell was able to perform all the viral infections in the absence of protein. The Tübingen group was unable to detect protein in their infectious RNA preparations by chemical methods. By serological techniques they showed that such preparations contained less than 0.02 per cent of TMV protein. The lesions resulting from viral infectious RNA inoculation appear within a shorter time than those evoked by the complete virus. On the other hand, isolated RNA is considerably less infectious than that of complete virus. The low infectivity upon direct assay is attributed to its susceptibility to plant ribonucleases. It must be pointed out in this connection that infectious TMV-RNA is highly sensitive to crystalline pancreatic ribonuclease action. The infectious capacity of pure viral RNA or its viral protein reconstitute previously digested by ribonuclease was lost. This finding is in striking contrast to similar experiments performed with intact virus particles.

The isolation of an infectious RNA from TMV stimulated similar investigations with some RNA-containing animal viruses. At the present state of our knowledge, the isolated nucleic acids from certain encephalitis viruses and two poliomyelitis viruses are reported to be infectious. All these infectious ribonucleic acids are sensitive in a similar way to crystalline pancreatic ribonuclease action, which is in accordance with the results obtained with infectious TMV-RNA. By contrast, ribonuclease has no effect on intact virus.

In conclusion, these viral phenomena reflect an enormous functional specificity of virus RNA, providing in turn the possible clarification of the more detailed molecular configuration of functionally active RNA and RNA genetics. The principal role in this interesting new branch of molecular biology will be played by coded RNA viruses or their biologically active nucleic acids. In order to increase the basic knowledge of the mechanism of action of these infectious viral nucleic acids, more work must be done in the field of structural, metabolic and genetic research. In these detailed studies the enzymes acting selectively, including especially ribonucleases of different specificity, may be most important.

REVIEW OF THE LITERATURE

CRYSTALLINE PANCREATIC RIBONUCLEASE

History. — Jones reported in 1920 a thermostable pancreatic enzyme capable of degrading ribonucleic acid (RNA) without releasing at the same time inorganic phosphorus or purine and pyrimidine bases (46, 47). In 1938 Dubos and Thompson purified Jones's enzyme and called it "ribonuclease" (28). Kunitz, in 1940, isolated this enzyme in crystalline form from the beef pancreas (55). It was found that small amounts of proteolytic enzyme may be retained as an impurity even in a crystalline enzyme. In 1948 McDonald described a method of preparing protease-free crystalline ribonuclease in which the isolation procedure incorporated a heating phase destroying the proteolytic enzymes. Protease-free ribonuclease is very important in critical work and especially in histochemical research (59). Anfinsen, in 1950, reported a method of preparating radioactive ribonuclease. It involved incubation of the pancreatic tissue before isolation proper in an atmosphere of $C^{14}O_2$, whereupon the radioactive carbon was incorporated with the enzyme protein (2).

Specificity. — Crystalline pancreatic ribonuclease is a highly specific phosphodieasterase which hydrolyses all known ribonucleic acids but has no effect on deoxyribonucleic acid. Loring, Carpenter and Roll (57) and Schmidt *et al.* (93) showed that this enzyme catalyses the cleavage only of certain strictly defined internucleotide bonds, namely those between the 3'-pyrimidine nucleoside phosphoryl groups and the 5'-hydroxyl groups of the adjacent purine or pyrimide nucleotide groups. Brown and Todd (20) demonstrated the specificity of crystalline pancreatic ribonuclease by employing as substrate 2'- and 3'-monoribonucleotide esters when only the pyrimidine nucleoside-3'-

alkyl phosphates were hydrolysed. Further support for the high specificity of ribonuclease came from the recent observations of Magrath and Brown (65). They used N-methyl uridine-3'-methyl phosphate as substrate and no hydrolysis occurred. This could mean that the enzyme is sensitive at least to an alteration of this kind in the pyrimidine base. Contrary observations have also been made. Mandel, Markham and Matthews (66) incorporated 2-thiouracil-S^{35} in lieu of uracil in the ribonucleic acid isolated from the mosaic virus of the tobacco plant. After ribonuclease digestion, thiouridylic acid was found to account for 37 per cent of the radioactivity.

Degradation products. — On account of its very great specificity, pancreatic ribonuclease reacts with ribonucleic acid to producec 3'-UMP, 3'-CMP and a series of oligonucleotides consisting of purine nucleoside units and terminated by a pyrimidine containing residue. These oligonucleotides were designated as limit polynucleotides by Schmidt *et al.* (93). The simpler components of the complex mixture of the limit polynucleotides of low molecular weights were successfully separated by Markham and Smith (75) by ionophoresis and by Volkin and Cohn (103) on ion exchange columns. They all have in common one structural property: each of these oligonucleotides contains one pyrimidine nucleotide group per molecule. The pyrimidine nucleotide group is always terminal and carries the secondary phosphoryl group of the chain on its 3'-carbon atom. Between 30 and 40 per cent of the purines of yeast ribonucleic acid and between 45 and 50 per cent of those of liver ribonucleic acid are found as oligonucleotides containing four or fewer mononucleotide groups after exhaustive digestion with ribonuclease. The remainder are present in the form of polynucleotides of higher order which have not been separated as yet. A considerable proportion of these oligonucleotides, corresponding to 15—20 per cent of the total phosphorus of the substrate, is not dialysable against water but dialyses readily against salt solutions. Until recently this fraction was considered to consist of polynucleotides of relatively high molecular weight which have been termed "cores" or "limit nucleic acids" by Zamenhof and Chargaff (105). This "ribonuclease resistant fraction" probably consist of a mixture of tetra- and pentanucleotides, each composed of purine

nucleotides with a terminal pyrimidine nucleotide, as proposed by Markham and Smith (73, 74, 75).

Reaction mechanism. — Brown, Dekker and Todd (19) and Markham and Smith (74) demonstrated the biphasic nature of the cleavage of RNA. The cleavage of internuclear bonds is not a simple hydrolysis for it does not take place until after intramolecular transphosphorylation. Cyclic-ended oligonucleotides and pyrimidine nucleoside-2' : 3'-phosphates originate first. They are then hydrolysed further into the actual end products: pyrimidine-3'-mononucleotides or mononucleotide groups. Heppel, Ortiz and Ochoa (38) have recently elucidated the quantitative aspects of the biphasic event by hydrolysing certain pyrimidine polynucleotides with pancreatic ribonuclease and comparing the consumption of enzyme between the cyclic terminal oligonucleotide degree and complete hydrolysis, i.e. the 3'-mononucleotide degree. If the aim is to produce complete hydrolysis in the same time as the cyclic terminal oligonucleotide phase takes, 1,500 times the amount of enzyme must be employed.

Synthesising effect. — Ribonuclease possesses synthetic capacities in certain conditions, as proved by Heppel *et al.* (39, 40). For instance, thanks to the catalysing property of ribonuclease, cytidine-3'-methyl phosphate has been produced from cytidine-2' : 3'-phosphate and methanol. Barker *et al.* (7) claimed that only primary alcohols are serviceable in the enzymic conversion of nucleoside cyclic phosphates into nucleoside alkyl phosphates.

Effect of pH. — The optimum pH of crystalline pancreatic ribonuclease was proved by Kunitz (55) to be in the region of pH 7.7. However, the RNA may undergo spontaneous degradation in this region and it is therefore often necessary to make the determinations at slightly acid pH values, between pH 5 and 6, where ribonuclease still possesses considerable activity.

Effect of temperature. — Kunitz (55) stated the optimum temperature of crystalline pancreatic ribonuclease at pH 5 to be + 65°C. Between pH 2 and pH 5 ribonuclease is very heat-stable. If it is heated in these conditions at + 100°C for 30 min. only ca. 20 per cent of its activity is destroyed.

Activators and inhibitors. — The optimum effect of pancreatic ribonuclease is obtained at an ionic strength of 0.1. No specific

activator is needed. No specific inhibitor is known. Many workers have assessed both the activating and inhibiting influence of univalent cations. Besides cations, a study has also been made of the effect of other substances on ribonuclease. Of the antibiotics, streptomycin and penicillin have been examined, of the basic dyes especially acridine. Heparin, deoxyribonucleic acid and various mononucleotides have been found to inhibit ribonuclease. McDonald (60) summarised these investigations well.

The protein character of pancreatic ribonuclease. — Crystalline ribonuclease is an albumin-type protein. Its elementary composition in per cent of dry weight is: C: 48.2; H: 6.2; N: 16.1; S: 3.6 (partly inorganic); P: trace; residue: 0.1 (55). Its amino acid composition is well known (15). The molecular weight of ribonuclease, calculated from sedimentation and diffusion data, is 13,000. Slightly differing values have been obtained depending on the technique used. The above figure was determined by Rothen (90). Smolens and Sevag (99) studied the antigenicity of ribonuclease and found the enzyme to be a very good antigen in spite of its low molecular weight. Anfinsen *et al.* (3, 4) studied the molecular structure of ribonuclease. Hirs, Moore and Stein (41) found that crystalline pancreatic ribonuclease is not homogenous. They were able with ion-exchange chromatography to isolate a major fraction "*a*" (the slower moving band) and a minor fraction "*b*". Hakin (36, 37) proved that what is customarily regarded as ribonuclease effect was in fact caused by fraction *a*. Fraction *b* had a different effect, forming chiefly 3'-GMP from ribonucleic acid, and 3'-UMP and 3'-CMP additionally. Ribonuclease fraction *a* catalyses a reversible exchange or transesterification reaction between cyclic terminal pyrimidine nucleotides and nucleosides. Ribonuclease fraction *b*, on the other hand, stimulates an irreversible dinucleoside monophosphate synthesis from the mixture of nucleoside and cytidine-2' : 3'-phosphate, uridine-2' : 3'-phosphate, or guanosine-2' : 3'-phosphate.

The use of pancreatic ribonuclease. — Crystalline pancreatic ribonuclease has been used mainly for two purposes. Brachet (13) described the histochemical ribonuclease test in which the tissue section is treated in the aqueous solution of ribonuclease to

remove RNA. After ribonuclease digestion, the parts of cell in which RNA was demonstrable prior to enzyme treatment cease to stain with basic dyes such as pyronine or toluidine blue. Nor do the same parts of the cell, as demonstrated by Davidson *et al.* (26), absorb ultraviolet light after the enzyme treatment whereas the staining of the chromatin of the cell nucleus e.g. by methyl green is unaffected. The method can consequently be used to localise ribonucleic acids in tissue sections. The other major use of ribonuclease has been in the elucidation of important structural features of ribonucleic acid, as shown by Todd and his colleagues and Markham and Smith in Great Britain, and Chargaff and Cohn in the USA.

Principles of assay of ribonuclease. — (a) Formation of acid-soluble degradation products from RNA: Mononucleotides and several oligonucleotides are acid-soluble, whereas RNA itself is precipitated on the addition of acid. On this is based the precipitation method which can be used in measuring RNA degradation. Hydrochloric, sulphuric and perchloric acids or glacial acetic acid have been the most common precipitants used. MacFadyen (61) reported on the uranyl chloride trichloracetic acid precipitant which readily precipitated undegraded RNA quantitatively in flocculent form from very dilute solutions even. Roth and Milstein (89) obtained an easily filtered precipitate by using an acid alcohol precipitant. The turbidimetric method reported by McCarty (58) is based on acid precipitation. In this method the undigested ribonucleic acid is precipitated as a very fine dispersion from dilute substrate solutions with aqueous acids. The formation of acid-soluble degradation products on the other hand can be assessed in the filtrates by phosphorus measurements or by ultraviolet spectrophotometry. Roth and Milstein (89) used P^{32}- labeled RNA as substrate, a method in which the course of RNA hydrolysis can be followed by radioactivity measurements of the filtrate.

(b) Formation of titratable acid groups: Kunitz (55) and Allen and Eiler (1) found titratable acid groups to originate in the course of RNA digestion. A proof of this was the gradual fall in the pH of the digest observable with the titrimetric technique. The method is too slow, especially for measuring the initial stages of hydrolysis, but its final extent can well

be measured by this method. Bain and Rusch (6) developed a method by which the acid groups liberated in the course of enzyme digestion could be determined manometrically in bicarbonate buffer. This method permits a very accurante study of the effect of ribonuclease from the initial stages of the hydrolysis. Zittle (106) employed this technique for kinetic studies of ribonuclease digestion.

(c) Formation of secondary phosphoryl groups: Since acid prostatic phosphatase serves as a specific hydrolysing agent for secondary hydroxyl groups it is possible to establish their presence enzymatically. Schmidt *et al.* (93) found that monoesterified phosphoryl groups of nucleotides were rapidly and completely hydrolysed by acid prostatic phosphatase, whereas diesterified phosphoryl groups were resistant to this enzyme. Up till now the phosphatase method has mainly been used for analysing ribonucleic digests in the final phase of hydrolysis.

(d) Method based on the absorption of ultraviolet light: Kunitz (56) found that the digestion of RNA with crystalline pancreatic ribonuclease was accompanied by a shift in the ultraviolet absorption spectrum of the substrate towards the shorter wavelengths. The shift in the ultraviolet spectrum was most distinct in the wavelength 290—305 mμ. On the other hand, hydrolysis of RNA with ribonuclease caused no appreciable optical changes at 260 mμ. This finding was of theoretical significance in that the degradation of RNA by alkalis to the mononucleotide stage is accompanied by a ca. 20 per cent increase in the ultraviolet absorption just at 260 mμ. This phenomenon is called the hyperchromic effect. The smaller absorption of RNA compared with the sum of the absorption of its free mononucleotides was ascribed to an alteration of the resonance behaviour of bases when they are bound in polynucleotides of relatively high molecular weight.

As the principal effect of ribonuclease is the degradation of ribonucleic acid to nucleotides, chiefly to pyrimidine mononucleotides, it is possible to follow the action of ribonuclease by means of nucleotide analysis.

(e) Paper chromatography in the analysis of nucleotides was developed and applied by Chargaff and his colleagues (21, 102, 103) and Markham and Smith (69, 71, 72). Paper chromatography

of nucleotides is based on the strong polarity of their phosphate groups so that they do not move in nonpolar solvent systems, as for instance in n-butanol saturated with water. On the other hand, their mobility can be accelerated by adding a quantity of water or other polar components or by suppressing the phosphoryl dissociation of nucleotides by adding acid to the solvent. There is now a host of different solvent systems to choose from for the paper chromatographic analysis of nucleotides and related substances (68, 84). The twodimensional approach is often necessary in analysing nucleotides by paper chromatography. A method commonly applied and found good is primary fractionation into zones and secondary analysis of the components of the zones by paper chromatography or other methods. The easiest way to localise nucleotides on chromatograph is to examine it either under an ultraviolet lamp with a high emission in the range of maximal nucleic acid absorption, thus visualising the spots as dark regions against background fluorescence of the paper or by the ultraviolet printing technique on photographic paper (44, 70). A limiting factor in ultraviolet light absorption is the quantity of material in question. The lower limit is ca. 5 μg nucleotides in a spot or zone. This, however, is partly dependent on the location of the absorption maximum. Digestion products liberated from ribonucleic acid by crystalline pancreatic ribonuclease have been succesfully analysed by the paper chromatography technique alone (73, 74, 75).

(f) Ion exchange chromatography for the analysis of nucleotides was developed chiefly by Cohn and his colleagues (22, 23). While paper chromatographic methods are generally used to analyse only very small quantities of material, even large amounts of material can be succesfully used in ion exchange chromatography. The best known of the anion exchangers employed are Dowex 1 and 2 and Amberlite IR 400 and IR 410 resins. Dowex 50 resin has been used extensively as a cation exchanger. These agents are very suitable for the separation of mononucleotides. Ion exchange column chromatography is well suited for separating nucleotides as the primary and secondary esters of phosphoric acid have a free acid function and are thus filtered through the cation exchanger but remain in the anion exchangers from neutral or slightly acid solutions. Anion exchange

affinity depends principally on the pH and ion concentration of the solution.

Paper chromatography is more rapid and easier to perform in practice, but for certain problems ion exchange column chromatography is invaluable in nucleotide analysis. This method has given very good results in the analysis of digestion products liberated from ribonucleic acid by crystalline pancreatic ribonuclease (103).

(g) Paper electrophoresis in the analysis of nucleotides was applied and developed by Markham and Smith (73) as a method to be used alone or combined with paper chromatography. Nucleotide isolation impossible to perform with paper chromatography or time-consuming with the ion exchange column chromatography is easily and rapidly performed by paper electrophoresis. All the analyses may be confined to small quantities, but the principal advantage of the method is perhaps that it makes possible the fairly accurate advance calculation of the relative mobilities of nucleic acid components, especially nucleotides, at a certain pH. This facilitates the identification of unknown compounds. The most suitable buffers are univalent buffers made of volatile substances and with non-volatile substances pervious to ultraviolet light. The salt content of the material under examination should be small. After the run the spots are localised on paper in the same way as in paper chromatography. The paper electrophoretic examination of ribonuclease digests is a very gratifying task as mononucleotides have a host of different ionising groups such as the primary and secondary acid groups of the phosphate groups, and the amino and enol groups of the bases. The enol groups of most bases are, however, dissociated at too high a pH and cannot be utilised, and are thus not important. The different dissociations of the phosphate groups and especially the amino groups, on the other hand, are of the greatest significance in the separation and identification of mononucleotides from one another. Crestfield and Allen (24) have studied the behaviour of mononucleotides and their isomers in different buffers and in different pH ranges. Edström (29) developed an electrophoretic micromethod which can be used to analyse the nucleic acid components contained in individual cells.

RIBONUCLEASES OTHER THAN PANCREATIC RIBONUCLEASE

The existence of other ribonucleases in addition to pancreatic ribonuclease may nowadays be taken for granted. They differ from pancreatic ribonuclease in regard to their heat lability and to their pH optima. In some cases highly purified enzyme fractions have been obtained, but with a few exceptions the specificity has not been delineated.

Animal ribonucleases. — Soluble extracts of different animal tissues have been obtained which exhibit a high degree of enzymatic activity upon ribonucleic acids. The distribution of rather unpurified enzymes in the tissues has been recorded by numerous investigators (6, 77, 89, 107).

More recently Kaplan and Heppel (50) reported a highly purified heat-stable enzyme from calf spleen. This fraction has been found to have exactly the same specificity as pancreatic ribonuclease. Ribonucleic acid is only partly degraded and pyrimidine mononucleotides are formed. A heat-stable ribonuclease with similar specificity has also been obtained from calf liver.

Roth (88) purified an alkaline and an acid ribonuclease from rat liver mitochondria. The first of these resembled pancreatic ribonuclease in its stability to heat and sulphuric acid treatment and may well have similar catalytic properties. Specificity studies on both fractions are to be published.

Rabinovitch and Dohi (86) reported a highly purified rat and guinea pig serum ribonuclease. They were found to be similar to crystalline pancreatic ribonuclease in their pH activity curve, thermostability and reaction to heparin. The investigation continues.

Finally, Teir and Kuusi (100), studied the tissue specificity of several animal ribonucleases.

Microbial ribonucleases. — Similar enzymatic activity was shown by a variety of micro-organisms. MacFadyen (61) reported that the aerobic sporebearing groups of Bacillus subtilis, B. mesentericus vulg. and B. megatherium were found to be agents for the rapid, intensive disintegration of ribonucleic acid. Woodward (104) studied the ribonuclease activity of Pasteurella pestis. Miller and Kozloff (81) studied the nucleic acid metabolism

of malarial parasites. McCarty (58) found that during growth in fluid media ribonuclease was produced by a variety of strains of group A hemolytic streptococci.

Studies on the production and properties of the ribonuclease in the culture filtrates of a soil Actinomyces (strain A) were described by Muggleton and Webb (83). Pancreatic and Actinomyces ribonucleases appeared to be similar in their action on gram-positive cells. The enzymes differ in their heat lability and possibly in their mode of action, since Actinomyces ribonuclease is able to hydrolyse the "core" of ribonucleic acid which is resistant to the pancreatic enzyme.

Kuninaka (53, 54) reported on the highly purified heat-stable ribonucleolytic enzyme system of Aspergillus oryzae. Its culture filtrates contain ribonucleases which depolymerise ribonucleic acid and liberate the four 3'-mononucleotides. Saruno (91) prepared a crystalline preparation with an acid pH-optimum, but it was not homogenous upon electrophoresis. More recently, two ribonucleases from the same source have been separated and partially purified. The first enzyme formed 3'-GMP rapidly from ribonucleic acid, but the other 3'-nucleotides were liberated exceedingly slowly. This fraction was heat-stable and showed a neutral pH optimum; a specific activity was achieved which approached that of pancreatic ribonuclease. The second fraction was also heat stabile with an acid pH optimum and, in contrast to the first enzyme, formed much more 3'-AMP and pyrimidine mononucleotides than 3'-GMP in the early stage of digestion of ribonucleic acid.

Plant ribonucleases. — Ribonuclease was recognised in the leaves, roots and seeds of plants by Jono (48, 49), and he examined in particular the products of the action of extracts from soya bean shoots on ribonucleic acid. Schlamowitz and Garner (92) extended this work and reported the nearly complete removal of phosphomonoesterase from the enzyme preparations.

Bredereck *et al.* (16, 17, 18) found that aqueous extracts of sweet almonds, lucerne seeds or pea sprouts contained an enzyme system capable of converting ribonucleate practically completely to the nucleosides and phosphoric acid, at a pH range between 5 and 6. He observed the almost quantitative precipitation of adenosine and guanosine after prolonged incubation of yeast

ribonucleic acid with extracts of sweet almonds. A fractionation of this system has so far not been attempted.

Axelrod (5) commented on the heat stability of the ribonuclease present in phosphatase preparations made from citrus fruit. These preparations were able to release 90 per cent of the total phosphorus of yeast ribonucleic acid as inorganic phosphate.

Bhimeswar and Sreenivasaya (10) partially purified the enzyme from castor bean sprouts.

Shuster and Kaplan (97) used barley and rye grass sprouts. More recently Shuster (96) purified 50-fold the ribonuclease from germinating rye grass and separated it from deoxyribonuclease and 3'-nucleotidase. Purified enzyme was observed to form mainly 5'-nucleotides from ribonucleic acid.

The present author (62) reported his preliminary studies concerning the action of several fungus extracts on yeast ribonucleic acid.

From pea and tobacco leaves, Holden and Pirie (42) purified a leaf ribonuclease. The leaf enzyme hydrolysed yeast ribonucleic acid more extensively than the pancreatic enzyme. There were also differences in thermostability and pH optimum and the leaf enzyme was more easily affected by several inhibitors. Markham and Strominger (76) investigated the specificity of leaf ribonuclease and found that it split all the internucleotide linkages of ribonucleic acid to yield nucleoside-2' : 3'-phosphates. The pyrimidine derivatives were inert, while purine derivatives were slowly hydrolysed to give nucleoside-3'-phosphates. This enzyme is an ideal tool for the production of cyclic pyrimidine nucleotides. Frisch-Niggermayer and Reddi (34) described another purification of ribonuclease from tobacco leaves and Reddi (87) investigated its specificity with similar results.

Bernheimer and Steele (9) studied ribonuclease distribution among higher plants. Aqueous extracts prepared from different parts of common plants were tested for ribonuclease activity and for capacity to inhibit pancreatic ribonuclease. Extracts of a few plants were found capable of degrading ribonucleic acid and a new, naturally occurring inhibitor of pancreatic ribonuclease was reported.

PRESENT STUDY

OBJECT

The purpose of the present study was to find out

I. whether ordinary fungi contain an enzyme degrading ribonucleic acid;

II. the optimum conditions for the activity of such an enzyme and its properties;

III. what degradation products are formed from the reaction of the enzyme with ribonucleic acid;

IV. whether, if ordinary fungi contain an enzyme degrading ribonucleic acid, this enzyme could be isolated and purified.

METHODS

ANALYTICAL METHODS

Fresh weight determinations were made from material air-dried at +25°C.

Dry weight determinations were made from material dried for 10 hours at +105°C.

Total nitrogen determinations were made by the micro Kjeldahl technique in the usual way without a catalyst from samples treated with H_2SO_4 using the equipment described by Markham (67). The microburette described by Scholander (94, 95) was used for titration. The method was controlled by determining the N-content of standard ammonium sulphate. This content was then calibrated to correspond to the amount of nitrogen in the samples analysed.

Total phosphorus was determined by the micro-determination method described by Berenblum and Chain (8). The period of shaking for the development of colour was 30 seconds. Extinction was measured against a blank solution treated in the same way. The absorption maxima of molybdenum blue were found to occur at 625 mμ and 730 mμ, and analysis measurements were made at the latter. They were performed with a P M Q II spectrophotometer, Carl Zeiss, Oberkochen/Württ., in a quartz cell 0.5 cm in diameter. The phosphate standard curve was determined by means of K_3PO_4 (Merck, p.a.) dried for 5 hours at +105°C and the measurements were made from samples containing 10, 20 and 30 μg of phosphorus. The phosphorum content of the analysis samples proper was then kept within these limits.

Paper electrophoresis technique was employed in the purification of lyophilised enzyme preparations. A semi-preparative apparatus of the author's own design was used for the purpose. The power unit employed was a source of current manufactured by Evans Electroselenium, Ltd., Harlow, Essex, voltage range 0—400, maximum strength of the current 12 mA. Veronal buffer pH 8.6 with an ionic strength of 0.05 M was used as the electrolyte. The paper used consisted of Whatman No. 1 and paper qualities by the same manufacturer for continuous electrophoresis. The run was varied as required.

Paper chromatography methods. — The ordinary single or two-dimensional paper chromatography technique was used for the study of ribonucleic acids and their derivatives, 5'-ribonucleotides, ribonucleosides, and purine and pyrimidine bases. The solvents used, the general chromatographic procedure, detection of spots, their quantitative elution and ultraviolet spectrophotometry are described in detail in the following. Finally, the accuracy of the determinations was assessed.

a) *Solvents.* — As far as possible, solvents were chosen for the following properties: non-absorption of ultraviolet light, not very susceptible to temperature fluctuations, and not requiring washed papers or equilibration of the paper with the vapour. Aliphatic alcohols were very suitable and were employed as acid, neutral and basic solutions. Used solvent was always employed to saturate the atmosphere of the tank.

Solvent No. 1: n-butanol/water, 86/14 (45, 69).

Solvent No. 2: n-propanol/conc.ammonia/water, 60/30/10 (27).

Solvent No. 3: tert.-butanol/constant boiling (at +26°C) hydrochloric acid/water, 70/13/17 (72, 98).

Solvent No. 4: ammonium sulphate-isopropanol/water, 79/2/19 (72).

Solvent No. 5: 0.1 M phosphate buffer pH 6.8/ammonium sulphate/n-propanol, 100/60/2 (11, 84).

Solvent No. 6: ethanol/1 M ammonium acetate, 7/3, pH 7.5 (84, 85).

Solvent No. 7: isobutyric acid/conc. ammonium hydroxide/water, 57/4/39, pH 4.3 (84, 51)

Solvents 1, 2, 3, 4 and 5 were the ones most commonly used in single dimensional chromatograms. Solvents 1 and 2 were used most generally in two-dimensional chromatography in the first stage of the run while the others, combined with 1 and 2 according to the test in question, were employed in the second stage. Two-dimensional chromatography analysis was applied for identification of some of the degradation products; solvent 1 was used for the first stage and, for the second stage, a minimum of three different solvents, mostly 5, 6 and 7, since 5'-ribonucleotides and ribonucleosides are well defined as regards these solvents (84).

b) *General technique.* — The majority of the chromatography runs were carried out in cylindrical glass tanks (50 cm × 22 cm) by the ascending technique. It was possible to run two cylindrical rolls of paper, one inside the other (55 cm × 45 cm and 40 cm × 45 cm), at a time. 300 ml of the solvent was measured into the bottom of the chambers. Ordinary aquarium-type tanks (60 cm × 60 cm × 35 cm) were employed in analyses involving descending chromatography, permitting the solvent to be run off the end of the paper. The props inside the chambers were of stainless steel, the urns of glass.

The known controls, intact RNA (Light & Co., twice purified), 5'-ribonucleotides, ribonucleosides (Pabst Laboratories) and the purine and pyrimidine bases (Krishell) were dissolved in Sörensen's phosphate buffer pH 7.0 to make stock solutions of the samples. Purine bases and purine ribonucleosides of poor solubility were dissolved in heated solutions, thus enabling their treatment analogously with other substances. The amount of control substance pipetted onto the starting points was generally 150—200 μg per spot.

Fungus enzyme hydrolysate of RNA was pipetted as such onto the sheet of chromatogram paper. Of the 242 species of fungi, Lactarius torminosus only was used as an enzyme in the chromatography analyses since it does not disturb ultraviolet photography of the chromatograms like many other more active species tend to do. A 1 per cent hydrolysate of RNA was pipetted onto the starting points: 15 μl in single dimensional runs and 25 μl in two-dimensional runs.

The paper used was Whatman No. 1, not pretreated at all. To speed up pipetting, hot or cold air was fan-blown as required onto the spot in question, care being taken to ensure that the temperature on the paper did not exceed +40 °C. After pipetting, the chromatogram sheets were sewn together along the edges with silk thread into cylindrical rolls which were placed on the bottom of the chamber in the solvent. The most important identifications runs with the ascending technique were performed in a thermostat at +27 °C. The orientation runs and runs using the descending technique were made at room temperature, ca. +20 °C.

c) *Development of the chromatograms.* — After the run, the sheets were dried overnight at room temperature. The spots were detected by ultraviolet printing technique on photographic paper (70). As nucleic acids nearly free from impurities or their even purer derivatives were used for the tests the source of light was a bactericidial 30 W lamp (Philips) though its light is not pure UV light. The results were controlled with the "Chromatolite" lamp as a special ultraviolet light source (a low pressure mercury vapour discharge tube with a quartz envelope, emitting approximately 90 per cent monochromatic radiation at a wavelength of 253.7 mμ, and a dark Chance's OX 7 filter. Hanovia, Ltd., Slough, Bucks.). No differences were observed. The chromatogram was used as a photographic negative and its image was printed on reflex document paper. The developing times varied greatly depending on the exposure times and the concentration of the substances to be examined. No staining was used to localise the spots.

d) *Elution and ultraviolet spectrophotometry.* — The following elution method was used with slight modifications for the quantitative determination of the various decomposition products contained in the hydrolysate (101, 102). In the development of the chromatograms care was taken to ensure that the ultraviolet photograph and the actual chromatogram could be matched exactly afterwards. This made it possible to note the exact position of the spots on the chromatograms, e.g. with a sharp needle. In addition, a square of millimetre paper was placed at the bottom to facilitate calculation of the area of the spot. Making use of the spot diagram perforated on millimetre paper, blanks were cut equal in area to the spots and at equal distances from the starting line, and were eluted and read at the same wavelengths as the corresponding spots. The variation of the blank values in the different parts of the chromatogram was thus observed. Both the spot proper and its control area were cut off and then, without touching by hand, cut up and placed in small 5 ml Erlenmeyer flasks. Extraction was performed with 5 ml of 0.1 N HCl in tightly sealed conical flasks by mechanical shaking for 5 hours at +37 °C. After extraction, the suspensions were neutralised with 0.1 N NaOH, filtered into sterile test tubes; the fluid from the powdered paper pulp was carefully pressed into a test tube with the aid of a glass rod. The eluates were then pipetted into suitable quartz cells and the extinction caused by them measured as such or from a suitable dilution of the eluates by a Zeiss P M Q II spectrophotometer. The measurement was made againts the solution employed. The ultraviolet spectra of both the actual spot and its blank eluate were measured between 215—320 mμ. The readings were made

at 5 mμ wavelength intervals; however, the maximum and minimum points were read as accurately as the equipment permitted. It was possible to make a preliminary identification of the substance under examination from the ultraviolet spectrum by comparing the spectrum with that of authentic control samples and by comparing the rf-values with one another. The concentration of the substance in question in the eluate, pH 7.0, was thus determined with the aid of known constants (84) on the basis of the maximum extinction readings which were, moreover, corrected in regard to blank extinctions.

The accuracy of the elution method was determined as far as possible in the experiments in which two-dimensional runs corresponding exactly to the hydrolysate analyses were made with authentic substances and in which the elutions and the ultraviolet measurements of the eluates were carried out in the same conditions. Table 8 and Fig. 35 give the results obtained. This gave the optimal concentration for the analyses and it was possible to use in the elution phase the coefficients of error calculated. Blank eluate material was obtained, furthermore, from a great quantity of chromatogram material treated in the same way. Fig. 33 gives the mean blank extinction values from a total of 65 determinations.

METHODS OF DETERMINING RIBONUCLEASE ACTIVITY

ACID PRECIPITATION METHODS

Determination of the precipitating part. — a) *The titration method* (25) was used to gain an approximate idea of the disintegrating effect on substrate of enzyme solutions diluted in a descending concentration. When tubes digested equally long are precipitated the limit at which enzyme influence ceases may be established. Comparison of the result with titres obtained from known substrate concentrations enables a rough estimation of the effect of the enzyme dilution used. The method is inaccurate as such, but it can be improved by measuring for instance the magnitude of the precipitated part by means of phosphorus analyses. It is naturally also possible to determine the acid-soluble phosphorus, but this requires knowledge of the enzyme's own phosphorus content and the primary acid-solubility of the substrate in regard to the precipitant used. The precipitant employed was MacFadyen's reagent in which 310 mg of uranyl acetate was dissolved in 100 ml of 10 per cent trichloracetic acid (61).

b) *The turbidimetric method* (58) is based on the precipitation of insoluble ribonucleic acid by 1 N HCl which makes it possible to measure the resultant very fine opalising precipitate turbidimetrically at 420 mμ. In certain conditions the method has been calibrated into a quantitative one with the best measuring moment occurring after adding the precipitant and the use of a substrate of non-variable acid-solubility. The observance of this procedure standardised certain transmission readings to correspondence with a known ribonucleic acid concentration. Enzyme influence was studied by the turbidimetric method as follows:

A 2 ml sample of the reaction mixture with a substrate concentration usually of 0.05 per cent was precipitated with the same amount of 1 N HCl. The precipitate was measured turbidimetrically at 420 mμ 20 min. after the addition of the precipitant against either distilled water or the corresponding blank sample with a Zeiss P M Q II spectrophotometer. The spontaneous hydrolysis of the substrate from the reaction mixture without enzyme was controlled at the same time. The measuring moment proved critical as the turbidity was not stable, changing in the course of time obviously because of the acid hydrolysis of the precipitated substrate (Fig. 2). This occurred with both commercial and highly purified RNA preparations. Considerable errors may also arise if the different acid-solubility values of the various substrates have not been determined in advance. The transmission values obtained with the turbidimetric method are illustrative only of the hydrolysis of the part not dissolved in 0.5 N HCl. The varying acid-solubility of different preparations in regard to each precipitant was not the only source of error. One and the same substrate was found to vary according to its concentration: the acid-solubility of the substrate seems to increase in inverse ratio to the concentration (Table 1).

To eliminate the above sources of error and to calibrate the turbidimetric method quantitatively, the substrate used was carefully purified twice. Its acid-solubility in different precipitants was determined and found faultless by chromatographic analysis. From the substrate thus prepared, solutions of different strength were made in concentrations usually approximating the concentration range of the substrate employed. These solutions were precipitated in the usual way and a curve was plotted for the transmission readings. The substrate concentration corresponding to each transmission values was read from this curve (Fig. 4). The amount of substrate degrading during enzyme digestion can thus be calculated.

Determination of the acid-soluble part: When ribonucleic acid is hydrolysed under the influence of an enzyme the amount of acid-soluble degradation products in the reaction mixture increases. When the insoluble portion of the ribonucleic acid is precipitated and filtered off it is possible by registering the increase in absorption at 260 mμ to measure the quantity of acid-soluble degradation products released in the filtrate. The precipitating reagent used was 0.75 per cent uranyl acetate — 25 per cent $HClO_4$ reagent ($HClO_4$, 70 per cent, specific gravity 1.67, Merck, p.a.). A 2 ml reaction mixture sample precipitated with 1/5 volume of the precipitant reagent was used most commonly. The mixture was filtered 10 min. after adding the precipitant. Absorption at 260 mμ was measured with a Zeiss P M Q II spectrophotometer from a suitable dilution of the filtrate, usually 0.5 : 5.0, against a blank solution (buffer-enzyme-precipitant) treated in the same way. The variation in the absorption values of the blank solutions was observed by determining in turn the absorption against distilled water and comparing the results with the means of the specific spectra of the components of the reaction mixture. The spontaneous hydrolysis of the substrate was observed from enzyme-free reaction mixture filtrates which were treated in the same way. The results were usually expressed as an increase in absorption at

260 mμ or as mere differences in the absorption (ΔE_{260}) in which case both the blank value and the 0 control of the substrate were deducted from the total absorption values. MacFadyen's precipitation reagent was not used in the measurement of acid-soluble degradation products as the absorption of thrichloracetic acid itself interferes with absorption measurements at 260 mμ.

Commercial ribonucleic acid (RNA, British Drug Houses, Ltd., London) was the substrate most commonly employed since the primary acid-solubility of substrate does not cause a direct error in the final results provided that the same preparation is used systematically. For the sake of comparison, parallel determinations were made with a better substrate (RNA, Light & Co., Ltd., twice purified), but the test results showed no deviations. Only the absorption values of the 0 moment were lower.

With isotope substrate (RNA-P^{32}, Abbot Laboratories, North Chicago, Ill., Oak Ridge, Tennessee) it was not possible to employ uranyl acetate perchloric acid reagent as the precipitant since the relatively pronounced radioactivity of uranyl acetate interferes especially in the initial phases of the reaction when the radioactivity of the filtrate is still low. The precipitation reagent used was acid alcohol precipitant, as recommended by Roth and Milstein (89), which gives an easily filtrable fine precipitate. The precipitant was prepared in the following way: 120 ml of distilled water was added to 80 ml of conc. HCl (c. 12.5 N HCl). The solution was diluted with 94 per cent ethyl alcohol to the final volume of 1000 ml. The normality of the solution was approximately 1 and its alcohol concentration 76 per cent. The 2 ml reaction mixture was usually precipitated with the corresponding quantity of acid alcohol. The increase in absorption at 260 mμ was measured from a suitable dilution of the filtrate against a blank sample. It was possible at the same time to compare the increase in radioactivity of the filtrate with the increase in absorption at 260 mμ in the course of digestion. The spontaneous hydrolysis of the substrate was followed in the same manner, using both the techniques, from a reaction mixture in which the enzyme was omitted. Owing to the low radioactivity of the isotope substrate (assay 0.18 mc/g) it was necessary to increase the concentrations of the reaction components to make possible an accurate direct comparison with inorganic phosphorus determinations. The scaler and ratemeter unit model FH 49, Frieseke & Hoepfner, G.M.P.H., Erlangen-Bruck, was used in the radioactivity measurements. An end window counter tube FHZ 15 with a window thickness 1.6 mg/sq. cm was used in the device.

The specific ultraviolet spectra of reaction mixture components. — A reaction mixture in which the final concentration of the substrate was 0.05 per cent and the final concentration of the fungus enzyme 0.1—0.2 per cent was the most commonly used mixture for registration of enzyme effect by spectrophotometric measurement of the increase at 260 mμ of the absorption caused by acid-soluble degradation products. The substrate was prepared in Michaelis buffer (78), usually in the proportion 2/5 volume of buffer in the reaction mixture. At the end of the reaction time precipitation was performed by adding 1/5 volume of uranyl acetate perchloric acid reagent. A 2 ml sample was thus

precipitated with 0.4 ml of the reagent. Absorption measurements were made mostly from the dilution 0.3 : 5.0 of the filtrate.

To obtain an idea of the specific spectrum of each component in the reaction mixture, absorption determinations were made at 260 mμ, imitating as faithfully as possible the reaction mixture conditions, from the following dilutions of reaction mixture components: RNA, 0.125 per cent, dilution 1 : 50, Armillariella mellea, 0.5 per cent, dilution 1 : 100, Michaelis buffer, dilution 1 : 50 and uranyl acetate perchloric acid reagent, dilution 1 : 100. The results of the spectrum measurements are analysed in Fig. 1. To the same end, the arithmetical sum of the above-mentioned dilutions of the reaction components was calculated and compared with the *in vitro* spectrum of the total reaction mixture. This spectrum was measured without precipitating the

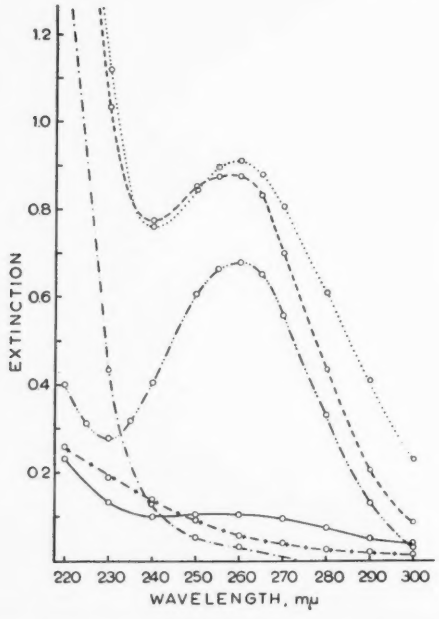

Fig. 1. — The specific ultraviolet spectra of the different components of the reaction mixture used in spectrophotometric measurements at 260 mμ; their arithmetic sum and, as points of comparison, the total spectrum *in vitro* of the real reaction mixture.

RNA, 0.125 per cent, diluted 1 : 50

Armillariella mellea, 0.5 per cent, diluted 1 : 100

Michaelis buffer, diluted 1 : 50

Precipitant reagent, diluted 1 : 100

The arithmetic sum of the absorption of the reaction mixture components in the above dilutions

The *in vitro* spectrum of the real reaction mixture

RNA, which is possible if the precipitant reagent is added to a reaction mixture ready diluted to the measuring volume. The figure shows that the proportion of enzyme, buffer and precipitant in total absorption is fairly small and definitely can not interfere with the absorption of the substrate at 260 mμ. Nor is there a great difference in the absorption maximum between the total spectrum of the real reaction mixture and the spectrum of the combined sum of reaction mixture components.

ULTRAVIOLET ABSORPTION METHOD

Kunitz (56) found that digestion of yeast ribonucleic acid by crystalline pancreatic ribonuclease is accompanied by a shift in the ultraviolet absorption spectrum of the substrate towards the shorter wavelengths. At the beginning of the digestion phase the decrease in absorption is linear to the enzyme concentration and from this the approximate correspondence between reaction time, enzyme concentration and extinction can be obtained from the equation:

$$-\frac{dE}{dt} = k \times c$$

In a wider range, the following formula was found to be better applicable to the concentration of ribonuclease:

$$-\frac{dE}{dt} = k \times c \times (E - E_f)$$

In it, E = the extinction at any time *(t)* in the course of the reaction and E_f = the final extinction. Integration of the former equation gives the following logarithmic expression:

$$-\log (E - E_f) = \frac{k \times c}{2.3} \times t - \log (E_o - E_f)$$

If $\log (E - E_f)$ is plotted as a function of the time *(t)*, a straight line is obtained the slope of which equals $-kc/2.3$ while $\log (E_o - E_f)$ is the intercept at $t = 0$.

It is possible from the foregoing to determine the activity of the ribonuclease used in terms of Kunitz units (KU). A unit of ribonuclease activity is defined by Kunitz (56) as the amount of enzyme which is capable of causing a decrease of 100 per cent per minute in $E_o - E_f$ of the substrate at +25 °C. $E_o - E_f$ is the maximum possible change in the extinction at 300 mμ when the substrate concentration is 0.05 per cent and pH of the reaction mixture is 5.0 in 0.05 M acetate buffer.

In practice, two methods can be employed to determine ribonuclease activity in Kunitz units per ml of enzyme solution. The readings can be plotted directly against t in minutes. The ribonuclease activity per ml of

solution is equal to the slope of the line drawn through the plotted points of E *versus t*. Dividing by the factor $E_o - E_f$ is necessary in order to express the activity in units per ml and make it comparable with that of the logarithmic expression. In the second method, the readings are plotted as log $(E—E_f)$ *versus t*. The ribonuclease activity per ml equals the slope of the line drawn through the plotted points of log $(E - E_f)$ *versus t*, multiplied by —2.3. The final result shows the activity per ml of the enzyme solution. To express activity in Kunitz units per mg of enzyme, the values obtained above are divided by the concentration of the enzyme solution.

Before examining the activity of fungus enzymes by means of this reaction, the method was calibrated and tested against commercial preparations. The substrate used was unpurified commercial ribonucleic acid (RNA, British Drug Houses, Ltd, London). Different dilutions of crystalline pancreatic ribonuclease (PRNase, Worthington Biochemical Corporation, Freehold, New Jersey, 1 × cryst., salt free) dissolved in 0.1 per cent gelatin solution were tested in order to find the optimal concentration. After experimentation, the final concentration arrived at was 0.001 mg/ml of pancreatic ribonuclease of the reaction mixture provided that the corresponding final concentration of the substrate in the reaction mixture was 0.5 mg/ml in 0.05 M acetate buffer at pH 5.0. The thermostat chamber of the spectrophotometer was set at +25 °C and the components of the reaction mixture preheated carefully to the same temperature. Determination was made by pipetting into a quartz cell 2 ml of substrate stock solution and 2 ml of enzyme stock solution. Extinction readings were taken at 300 mμ every minute for about 10 minutes, depending on the rate of drop in extinction. The initial value E_o was determined through extrapolation and the final value E_f several hours after there was no observable change in the extinction. Figs. 37 a and 37 b and Table 10 give the results of the activity determinations of pancreatic ribonuclease.

The activity determinations of the fungus enzymes used were made by this method, observing carefully the conditions stated above. It was easy to compare enzyme preparations at different stages of purification with the activity value for crystalline pancreatic ribonuclease. This gave a good idea of the distribution of the activity of different preparations into various purification fractions. For accuracy, the method demands careful calibration in regard to the enzyme and substrate in order to obtain a suitable rate of reaction. The reliability of the slope is sensitive to a reaction rate that is too rapid or too slow. When this occurs the result is usually reduced activity of a weight unit of the enzyme preparation as the enzyme concentration grows. The reaction was regarded as acceptable if a minimum of 3 points was obtained on the straight line during the first 10 minute period of digestion.

MATERIAL

RIBONUCLEIC ACIDS

Commercial ribonucleic acids. — Commercial ribonucleic acid preparations were used as substrate for the study of ribonuclease activity provided the impurity content, e.g. acid-soluble degradation products, was not the direct cause of false results. This depended, again, to a great extent on the method used and the object investigated. On the basis of preliminary tests with available commercial preparations, two preparations were accepted for use as such: RNA, British Drug Houses, Ltd., London, and RNA, Light & Co., Ltd., Poyle—Colnbrook—Bucks. These preparations were not beyond criticism, as the chromatographic analyses showed, and their acid-solubility was great. They were, however, considered serviceable with certain reservations.

Purified ribonucleic acid. — To produce an ideal ribonucleic acid preparation a host of commercial preparations made by different factories was examined. Various purification methods were tried experimentally to obtain an idea of the character and properties of each preparation. In the selection of the final purification method and the most suitable preparation attention was paid to the following points: the complexity of the purification method, the difficulties involved in the handling of the preparation during purification, the yield, the acid-solubility of the purified preparation and the chromatographic reliability of the purified preparation.

The tests showed that RNA, Light & Co., Ltd. was the preparation most easy to purify conclusively so as to meet even high requirements. The purification method (43, 104) was as follows: 10 g of the commercial preparation was suspended in 50 ml of distilled water. The suspension was dialysed against running tap water for 24 hours at room temperature with a magnetic mixer inside the dialysis membrane all the time. The same suspension was then dialysed in the same conditions against distilled water for 24 hours at +6 °C, changing the water at 5-hour intervals. After dialysation, the ribonucleic acid was dissolved completely by adding to it a minimal quantity of 5 N NaOH and ensuring that the pH did not rise above 6. The ribonucleic acid dissolved in the solutions was then precipitated with five times the volume of glacial acetic acid at room temperature for 10 min. The precipitate was filtered rapidly by suction on filter paper. The sediment was washed twice with a five-fold quantity of glacial acetic acid, then three times with 94 per cent ethanol and lastly three times with ether. The sediment was finally dried overnight in a vacuum exsiccator on NaOH. The preparation thus obtained was then treated a second time in exactly the same way inclusive all its intermediate phases.

No difficulties of handling were experienced by the purification method. The yield was 28 per cent and the preparation obtained was relatively soluble in the solvents used. The acid-solubility of the preparation in 1 N HCl and uranyl acetate-perchloric acid reagent is shown in Table 1. The preparation was chromatographically blameless in several solvent systems. The phosphorus content of the preparation was 7.31 per cent, all in organic form.

Isotope ribonucleic acid. — Radioactive ribonucleic acid (RNA—P^{32}, Abbot Laboratories, North Chicago, Ill., Oak Ridge, Tennessee, U.S.A.) was used partly in experiments with the liberation of inorganic phosphorus. The specific radioactivity of the preparation was 0.18 mc/gm (Lot No. 1045—122). It was not possible to study the acid-solubility of the preparation because of the shortage of supplies. On the other hand, it was ideal for chromatographic studies.

RIBONUCLEIC ACID DERIVATIVES

Purine and pyrimidine bases. — In chromatography analyses the following Krishell's purine and pyrimidine bases were used as such as controls: adenine, guanine, cytosine and uracil.

Ribonucleosides. — The following ribonucleosides made by Pabst Laboratories (Pabst Brewing Company, Milwaukee, Wisconsin) were used in the chromatography analyses: adenosine, guanosine, cytidine, and uridine. Table 6 and Figs. 25, 26, 27 give the results of the chromatographic analyses. The ultraviolet spectra are shown in Fig. 31.

5'-ribonucleotides: The following 5'-ribonucleotides made by Pabst Laboratories were used in the chromatography analyses: AMP, ADP, ATP, GMP, GDP, GTP, IMP, IDP, ITP, CMP, CDP, CTP, UMP, UDP, UTP. Table 6 and Figs. 28, 29, 30 give the results of the chromatographic analyses.

ENZYMES

Fungus enzymes. — Table 2 lists 242 different fungus species. The ribonucleolytic activity of these species was studied from crude water extracts made of air-dried material. The Armillariella mellea and Lactarius torminosus fungi were selected for further study. Their ribonuclease activity was examined in different conditions and in the different purification phases. For the chromatographic analyses only Lactarius torminosus fungus enzyme was used as it causes no unspecific absorption in ultraviolet photography.

Crystalline pancreatic ribonuclease. — Crystalline pancreatic ribonuclease (PRNase, Worthington Biochemical Corporation, Freehold, New Jersey, 1 × crystallised, salt-free preparation) was used in the present investigation solely as a control enzyme and for calibration of methods. The specific activity of the enzyme was 46.5 Kunitz units. Figs. 37 a and 37 b show the specific activity and the values obtained are given in Table 10.

TABLE I

THE ACID-SOLUBILITY OF TWICE PURIFIED RIBONUCLEIC ACID (LIGHT & CO., LTD.) IN 1 N HCL AND 0.75 PER CENT URANYL ACETATE — 25 PER CENT PERCHLORIC ACID REAGENT.

THE 0.1 PER CENT AND 0.05 PER CENT RNA SOLUTIONS WERE PRECIPITATED AT +20 °C WITH THE SAME VOLUME OF 1 N HCL. 2 ML OF THE SAME SOLUTIONS WAS PRECIPITATED WITH 1/5 VOLUME OF THE PERCHLORIC ACID REAGENT AT +20 °C. EXTINCTION WAS DETERMINED FROM THE FILTRATES AT 260 mμ, ALSO TOTAL PHOSPHORUS. THE RESULTS WERE CALCULATED IN PER CENT OF TOTAL EXTINCTION AND TOTAL PHOSPHORUS.

Extinction at 260 mμ (Per Cent of Total Extinction)				Acid Soluble Phosphorus (Per Cent of Total Phosphorus)	
Hydrochloric Acid Solubility		Uranyl Acetate-Perchloric Acid Solubility		Hydrochloric Acid Soluble	Uranyl Acetate-Perchloric Acid Soluble
RNA 0.1 per cent	RNA 0.05 per cent	RNA 0.1 per cent	RNA 0.05 per cent	RNA 0.1 per cent	RNA 0.1 per cent
24	35	6	13	20	5

THE DEGRADING EFFECT OF CRUDE FUNGUS EXTRACTS ON RIBONUCLEIC ACID

METHOD AND MATERIAL

Method in general. — The method applied was the turbidimetric method in which the transmission values were measured at 420 mμ 20 minutes after the addition of the precipitate. The method was checked and calibrated against crystalline ribonuclease. The method was developed quantitatively by determining experimentally in standard conditions the transmission values corresponding to the different substrate concentrations.

Enzymes. — The investigation was concerned with the 242 different fungus species shown in Table 2. After collection and identification the fungi were cut up and air-dried at +25 °C on metal netting, crushed mechanically into dust-like powder and kept in plastic bags. The term fungus powder is used here for the raw material thus treated and stored. It was the original material in all phases of the work. Solutions were made of the different fungus powders by weighing 100 mg into a test tube. 4.9 ml of distilled water was added and the mixed suspension was incubated for 1 hour at +37 °C in a water bath. After incubation the suspension was filtered through Whatman No.5 filter paper. A more or less coloured but clear solution was obtained through extraction from all the fungus samples and its activity was measured undiluted.

The crystalline ribonuclease concentration of the stock solution was 1 mg/ml of distilled water containing 0.1 per cent of gelatin. The working solution was prepared by diluting the stock solution with water as required. The final enzyme concentration in the reaction mixture at pH 7.8 was 0.5 μg/ml. Crystalline enzyme was used to control the method and calibrate the concentrations of the solutions.

Substrate. — Purified ribonucleic acid of Light & Co., Ltd. was used. For crystalline ribonuclease, a 0.2 per cent RNA solution was prepared in Michaelis buffer, final pH 7.8. When necessary, the pH was adjusted more accurately at a suitable point by adding 0.1 N HCl. A 0.1 per cent RNA solution was prepared in Michaelis buffer giving a final pH of 4.0 (62) for the fungus enzyme. Dissolution was performed by adding the smallest possible amount of 0.1 N NaOH to the RNA, after which the solution was fully clear.

EXPERIMENTAL PROCEDURE AND RESULTS

FACTORS AFFECTING THE TURBIDITY REACTION

Deciding on the measuring time proved to be important. To observe the spontaneous hydrolysis of the precipitated substrate, substrate solutions of 0.02 per cent, 0.06 per cent and 0.12 per cent were made by diluting the stock solution. The solutions were precipitated in a cell 0.5 cm in diameter by adding an equal volume of 1 N HCl. Turbidity fluctuations were followed for 6 hours by measuring the transmission values at 420 mμ against distilled water. Fig. 2 shows the effect of the measuring time on transmission readings tested with three different substrate concentrations. Transmission diminished at first and was at its minimum 20 min. after the addition of the precipitant. The transmission values then began to climb, obviously because of the acid hydrolysis of the precipitated RNA. The best way to obtain comparable results was to make the turbidity measurements 20 min. after adding the precipitant. This was the procedure applied consistently when this method had to be used.

It is always important to know the degree of acid-solubility of the substrate as transmission changes illustrate only the hydrolysis of the part not dissolved in 0.5 N HCl. The acid-solubility of ribonucleic acid preparations was studied in connection with their purification (Table 1). In order to avoid fluctuation in acid-solubility, the same purified RNA preparation of Light & Co., Ltd. was always employed in the activity determinations of crude fungus extracts. The HCl solubility of the preparation was 24 per cent in a 0.1 per cent concentration.

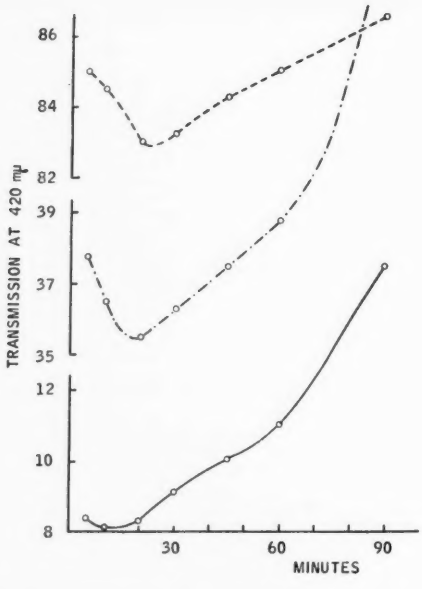

Fig. 2. — The effect of measuring time on turbidity readings in the precipitation of ribonucleic acid solution of different strength with 1 N HCl.

0.5 N HCl + 0.01 per cent RNA
0.5 N HCl + 0.03 per cent RNA
0.5 N HCl + 0.06 per cent RNA

CALIBRATION OF THE TURBIDITY REACTION

This was performed with crystalline ribonuclease (concentration in the reaction mixture 0.001 mg/ml) at pH 7.8 and 4.0 using a 0.2 per cent RNA concentration. Identical parallel determinations were made simultaneously with both active (Lactarius torminosus) and fairly active (Tylopilus felleus) fungus extract with an RNA concentration of 0.1 per cent and pH 4.0.

The following solutions pre-heated in the same conditions for 10 min. in a water bath at +37 °C were pipetted into graduated flasks:

Reagent:	*Test:*	*Blank:*	*Control:*
Substrate:			
(a) RNA, 2 mg/ml, pH 7.8	10 ml	—	10 ml
(b) RNA, 1 mg/ml, pH 4.0	10 "	—	10 "

	Test:	*Blank:*	*Control:*
Enzyme:			
(a) Crystalline PRNase 0.001 mg/ml	10 ml	10 ml	—
(b) Fungus extract 1:10 (Lactarius torminosus resp. Tylopilus felleus)	10 "	10 "	—
Buffer:			
(a) Michaelis buffer pH 7.8	—	10 "	10 ml
(b) Michaelis buffer pH 4.0	—	10 "	10 "

The 2 ml samples taken at suitable time intervals were precipitated by adding 2 ml of 1 N HCl. 20 min. after adding the precipitant to the reaction mixture the transmission values were measured against a blank solution precipitated in the same way in a cell 0.5 cm in diameter. If the control solution was precipitated simultaneously the substrate was not found to hydrolyse spontaneously.

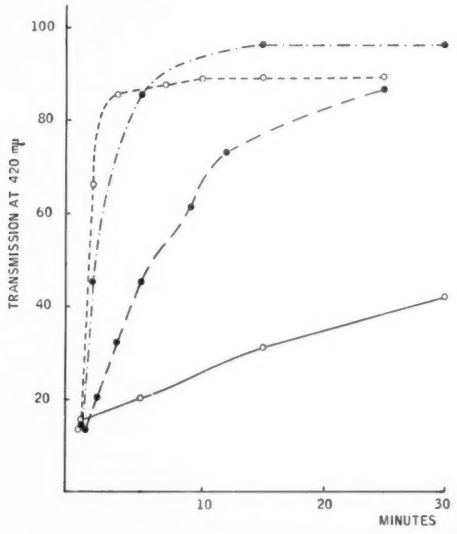

Fig. 3. — Hydrolysis of ribonucleic acid with active and weakly active fungus extract (1 : 10) compared with the effect of crystalline pancreatic ribonuclease.

- Active fungus extract (Lactarius torminosus) at pH 4.0
- Weakly active fungus extract (Tylopilus felleus)at pH 4.0
- Crystalline PRNase (reaction mixture concentration 0.001 mg/ml) at pH 4.0
- Crystalline PRNase (reaction mixture concentration 0.001 mg/ml) at pH 7.8

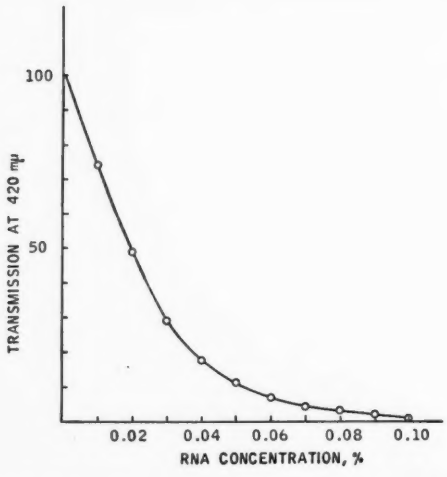

Fig. 4. — Transmission values as a function of the RNA concentration

Fig. 3 gives the results of the degradation of RNA by crystalline PRNase and fungus extracts as a function of the reaction time. Observation of the enzymatic hydrolysis of ribonucleic acid showed that the reaction was completed within 10 min. in optimal conditions if the concentrations of the substrate and enzyme solutions were in the above-mentioned ratios. A reaction time of 5 min. was consequently used for the material introduced in Table 2 in order to emphasis the activity differences of the crude fungus extracts. To calculate the RNA concentration corresponding to the transmission readings, a dilution series was made from RNA in buffer with concentrations ranging from 0.10 per cent to 0.01 per cent. 2 ml samples were precipitated with 2 ml of 1 N HCl. The transmission values were measured against a buffer-HCl control 20 min. after adding the precipitant in cells 0.5 cm in diameter. The results recorded were the means of three determinations. Fig. 4 shows the transmission values as a function of the RNA concentration. The substrate concentration corresponding to the transmission reading can be determined graphically from this curve.

DETERMINATION OF THE ACTIVITY OF CRUDE FUNGUS EXTRACTS

The following solutions pre-heated in the same conditions for 10 min. in a water bath at +37 °C were pipetted into test tubes (10 × 100 mm):

Reagent:		*Test:*	*Blank:*	*Control:*
Substrate:	RNA, 1 mg/ml, pH 4.0	1 ml	—	1 ml
Enzyme:	Fungus extracts (given in Table 2) in 1 : 10 distilled water	1 "	1 ml	—
Buffer:	Michaelis buffer pH 4.0	—	1 "	1 "

The solutions were mixed at the beginning and end of incubation. The reaction time was 5 min., whereupon 2 ml of 1 N HCl was added to the tubes. The pH of the reaction mixture was 4.0—4.2. After the addition of the precipitant the tubes were removed from the water bath. The next mixing of the solutions and the turbidity determination against a blank solution were made 20 min. after the addition of the precipitant in cells 0.5 cm in diameter. Two readings were taken and their mean calculated. A spectrophotometric check was made between the readings. The transmission values of the blank solutions were then determined against distilled water. To follow the spontaneous hydrolysis of the substrate, the turbidity of the control solution was measured at the beginning and end of the reaction. The transmission values of the control solutions, however, remained the same for 25 min. from the beginning of incubation. 4 samples were examined at a time and the time limits given were adhered to strictly. To calculate the percentual hydrolysis of the substrate, the substrate concentration corresponding to the transmission values was determined graphically from the curve (Fig. 4). The quantity of RNA degraded under the influence of the fungus extract was then calculated in per cent of the RNA present in the reaction mixture at the beginning of incubation. Table 2 gives the quantitative results of the ability of 242 different fungus extracts to degrade ribonucleic acid in the carefully standardised test conditions described above. The transmission value illustrates the hydrolysis of the substrate solution digested in 5 min. with every fungus extract. From this was calculated the total percentage of HCl-soluble degradation products originating during the reaction.

TABLE 2

A LIST OF THE FUNGI EXAMINED BY THE PRESENT AUTHOR WITH REGARD TO THE DEGRADING EFFECT ON RIBONUCLEIC ACID.

No.	Name and Grouping of the Fungi	Transmission at 420 mμ	Per cent degradation of substrate
	MYXOMYCETES:		
	Nomenclature according to Fries (33).		
1	Badhamia capsulifera	78.5	83
2	Lycogala epidendrum	36.0	48
	ASCOMYCETES:		
	Nomenclature according to Migula (80).		
3	Gyromitra esculenta	28.0	38
4	,, infula	41.5	53
5	Helvella macropus	20.0	26
6	Otidea leporina	25.0	35
7	,, onotica	20.0	26
8	Aleuria aurantia	24.0	32
9	Peziza badia	28.0	38
10	Rhytisma acerinum	19.0	24
	BASIDIOMYCETES:		
	Aphyllophorales, etc.:		
	Nomenclature according to Bourdot and Galzin (12) and Kühner and Romagnesi (52).		
11	Calocera flammea	92.0	94
12	Sparassis crispa	15.0	14
13	Clavaria botrytes	71.0	78
14	,, flava	49.5	60
15	,, abietina	46.0	58
16	,, cinerea	30.0	41
17	,, fusiformis	50.0	62
18	,, pistillaris	32.5	44
19	,, ligula	22.0	30
20	Merulius tremellosus	82.0	86
21	Dryodon coralloides	76.5	81
22	,, cirrhatum	46.0	58
23	,, corrugatum	44.0	56

No.	Name and Grouping of the Fungi	Transmission at 420 mμ	Per cent degradation of substrate
24	Hydnum repandum	34.5	46
25	„ rufescens	48.0	60
26	Sarcodon imbricatum	90.5	92
27	Calodon caeruleum	12.0	5
28	„ aurantiacum	60.5	70
29	„ ferrugineum	15.5	14
30	„ cyathiforme	14.0	12
31	Polyporus ovinus	37.0	49
32	Leucoporus arcularius	67.5	75
33	Leptoporus amorphus	15.0	14
34	„ adustus	17.0	20
35	Phaeolus Schweinitzii	40.0	52
36	Coriolus hirsutus	16.0	17
37	„ zonatus	16.0	17
38	„ abietinus	68.0	75
39	„ connatus	19.0	24
40	Lenzites betularia	19.0	24
41	„ saepiaria	85.0	88
42	Trametes cinnabarina	19.0	24
43	„ odorata	50.0	62
44	Ungulina marginata	33.5	45
45	„ fuliginosa	58.5	68
46	Ganoderma applanatum	25.5	35
47	Xanthochrous perennis	60.0	69
48	„ radiatus	80.0	84
49	Cantharellus cornucopioides	36.0	48
50	„ tubaeformis	50.0	62
51	„ cibarius	39.0	51
	Agaricales:		
	Nomenclature according to Moser (82).		
52	Gyroporus castaneus	35.0	47
53	„ cyanescens	47.0	59
54	Suillus Grevillei	49.0	60
55	„ luteus	65.0	73
56	„ bovinus	94.0	95

No.	Name and Grouping of the Fungi	Transmission at 420 mμ	Per cent degradation of substrate
57	Suillus variegatus	70.0	77
58	„ piperatus	90.0	92
59	Leccinum testaceo-scabrum	40.0	52
60	„ scabrum	49.5	60
61	„ scabrum var. niveum	64.0	72
62	Boletus edulis	91.0	93
63	„ edulis f. pinicola	95.0	96
64	Tylopilus felleus	28.0	38
65	Xerocomus badius	77.0	82
66	„ subtomentosus	85.5	88
67	Paxillus involutus	14.0	12
68	„ atrotomentosus	18.0	22
69	Gomphidius glutinosus	70.0	77
70	„ roseus	38.0	50
71	„ rutilus	51.5	62
72	Hygrophorus Karstenii	58.0	68
73	„ piceae	79.0	84
74	„ cossus	61.0	70
75	„ hypothejus	87.0	90
76	„ olivaceo-albus	70.5	77
77	„ erubescens	86.5	89
78	„ agathosmus	55.0	65
79	„ camarophyllus	78.0	83
80	Camarophyllus pratensis	83.0	87
81	Lyophyllum ulmarium	68.0	75
82	„ connatum	56.0	66
83	„ conglobatum	20.0	26
84	Hygrophoropsis aurantiaca	93.5	95
85	Clitocybe nebularis	81.0	85
86	„ odora	63.0	71
87	„ clavipes	40.0	52
88	„ infundibuliformis	35.0	47
89	„ gilva	88.0	91
90	„ rivulosa	31.0	43
91	„ obsoleta	77.0	82
92	„ fragrans	67.5	75
93	„ angustissima	22.0	30

No.	Name and Grouping of the Fungi	Transmission at 420 mμ	Per cent degradation of substrate
94	Clitocybe diatreta	16.0	17
95	,, dicolor	18.0	22
96	,, brumalis	43.5	55
97	Laccaria amethystina	24.0	32
98	,, proxima	20.0	26
99	Tricholomopsis rutilans	29.0	40
100	Collybia peronata	60.0	70
101	,, confluens	54.0	65
102	,, acervata	56.0	66
103	,, dryophila	60.0	70
104	,, butyracea	73.5	79
105	,, maculata	58.0	68
106	Armillariella mellea	91.0	93
107	Tricholoma flavobrunneum	49.0	60
108	,, albobrunneum	57.0	67
109	,, vaccinum	47.0	59
110	,, imbricatum	40.0	52
111	,, portentosum	93.0	95
112	,, flavovirens	98.0	99
113	,, inamoenum	94.0	95
114	,, album	60.0	69
115	,, columbetta	74.0	80
116	,, saponaceum	90.0	92
117	,, virgatum	50.0	62
118	Cantharellula umbonata	83.0	87
119	,, cyathiformis	19.5	24
120	Lentinellus cochleatus	80.0	84
121	Pleurotus ostreatus	67.5	75
122	,, ostreatus var. pulmonarius	45.0	57
123	Panellus mitis	37.0	49
124	Hohenbuehelia serotina	47.0	59
125	Flammulina velutipes	86.0	89
126	Marasmius scorodonius	94.0	95
127	,, oreades	65.0	73
128	Mycena metata	38.0	50
129	,, galericulata	40.0	52
130	,, rosella	45.0	57
131	,, pura	80.0	84

No.	Name and Grouping of the Fungi	Transmission at 420 mμ	Per cent degradation of substrate
132	Mycena alcalina	43.0	55
133	,, epipterygia	33.0	45
134	,, viscosa	94.0	95
135	,, vulgaris	40.0	52
136	Lepista nuda	78.0	83
137	Clitopilus prunulus	87.5	90
138	Pluteus cervinus	94.0	95
139	Amanita vaginata	50.0	62
140	,, muscaria	82.0	86
141	,, muscaria f. aureola	80.0	84
142	,, citrina	91.0	93
143	,, porphyrea	95.0	96
144	,, rubescens	82.0	86
145	Macrolepiota procera	52.0	62
146	,, rhacodes	75.0	80
147	Lepiota cristata	93.0	95
148	,, clypeolaria	82.0	86
149	Agaricus vaporarius	19.0	24
150	,, arvensis	21.0	28
151	,, abruptibulbus	29.0	40
152	Cystoderma amianthinum	81.0	85
153	,, carcharias	27.0	37
154	,, granulosum	22.0	30
155	Tubaria sp.	45.0	57
156	Rozites caperata	25.0	35
157	Hebeloma fastibile	76.0	81
158	,, crustuliniforme	84.0	88
159	Inocybe geophylla	18.0	22
160	,, asterospora	15.0	14
161	Alnicola escharoides	19.0	24
162	Gymnopilus liguiritiae	34.0	46
163	,, penetrans	47.5	59
164	Dermocybe cinnamomea	92.0	94
165	,, semisanguinea	45.0	57
166	,, sanguinea	70.0	77

No.	Name and Grouping of the Fungi	Transmission at 420 mμ	Per cent degradation of substrate
167	Cortinarius gentilis	25.0	35
168	Hydrocybe armillata	34.0	46
169	,, bivela	36.5	48
170	,, hoeftii	75.0	80
171	,, brunnea	26.5	36
172	,, flexipes	20.0	26
173	,, obtusa	30.0	41
174	Myxacium elatius	24.0	32
175	,, collinitum	22.0	30
176	,, mucosum	39.5	51
177	Phlegmacium triumphans	41.0	53
178	,, fulgens	14.0	12
179	,, traganum	85.0	88
180	,, alboviolaceum	18.0	22
181	,, camphoratum	50.0	62
182	,, bolare	16.0	17
183	,, pholideum	67.0	75
184	,, anomalum	15.0	14
185	Leucocortinarius bulbiger	93.5	95
186	Conocybe tenera	24.0	32
187	Pholiota heteroclita	74.0	80
188	,, squarrosa	50.0	62
189	,, flammans	75.0	80
190	,, spumosa	69.0	76
191	,, astragalina	25.0	35
192	,, alnicola	78.0	83
193	,, flavida	27.5	37
194	Kuehneromyces mutabilis	35.0	47
195	Naematoloma capnoides	83.0	87
196	,, sublateritium	65.0	73
197	Stropharia Hornemannii	47.0	59
198	,, aeruginosa	58.0	68
199	,, cyanea	30.0	41
200	,, semiglobata	25.0	35
201	Panaeolus campanulatus	16	17
202	Psathyrella candolleana	19.0	24
203	,, fusca	18.0	22
204	,, spadicea	33.0	45

No.	Name and Grouping of the Fungi	Transmission at 420 mμ	Per cent degradation of substrate
205	Coprinus comatus	31.5	43
206	,, atramentarius	52.0	62
207	,, domesticus	32.0	44
208	,, micaceus	34.5	46
209	,, disseminatus	15.0	14
210	Russula adusta	31.0	43
211	,, foetens	77.0	82
212	,, vesca	96.5	97
213	,, aeruginea	27.0	37
214	,, claroflava	61.0	70
215	,, decolorans	30.0	41
216	,, xerampelina	24.0	32
217	,, puellaris	20.0	26
218	,, paludosa	22.0	30
219	,, emetica	27.0	37
220	,, fragilis	30.0	41
221	Lactarius vellereus	86.0	89
222	,, torminosus	80.0	84
223	,, resimus	56.0	66
224	,, repraesentaneus	73.0	79
225	,, aspideus	62.5	71
226	,, deliciosus	63.5	71
227	,, necator	69.0	76
228	,, vietus	67.0	75
229	,, trivialis	97.0	98
230	,, mammosus	84.5	88
231	,, mitissimus	80.0	84
232	,, helvus	25.0	35
233	,, camphoratus	32.0	44
234	,, rufus	31.0	43
235	,, thejogalus	55.0	65
	Gastromycetales:		
	Nomenclature according to Fries (33).		
236	Bovista nigrescens	18.0	22
237	Lycoperdon nigrescens	88.0	91
238	,, perlatum	88.0	91
239	,, polymorphum	94.0	95
240	,, pyriforme	30.0	41
241	,, umbrinum	88.0	91
242	Calvatia saccata	20.0	26

DISCUSSION

Methodical errors, those caused by the reagents and errors due to the material must be distinguished from the factors of error occurring in the examination of the fungus material.

Methodical errors were discussed in connection with calibration and controls. As the turbidity reaction was fairly labile special attention was paid to factors that might diminish its sensitiveness or accuracy. Very carefully determined standard test conditions were established to ensure comparability of the results. To control the reliability of the results, 50 samples of the fungus material were re-tested. No significant deviations were observed on comparing the results. In the same conditions and always performed in the same manner the turbidity reaction proved quite a reliable method and has been employed extensively in other investigations for this reasons.

To eliminate errors due to the reagents, NaCl was omitted from the Michaelis buffer series for the obvious risk of inhibition. The ion strength of the buffer was subsequently 0.023 M in the final reaction mixture dilution.

Errors due to the material were caused partly by the substrate employed, partly by the enzyme material studied. Great attention was paid to the purification of the substrate and the study of its properties. The observations made have already been reported. The same RNA preparation (Light & Co., Ltd., twice purified) was used consistently in examinations of the activity of the fungus material, and its properties were charted as accurately as possible.

The principal potential error of the fungus material was erroneous identification. Identification, however, was made immediately after collection from fresh material, thus reducing the risk of making a mistake. Special attention was paid to rarer species and in doubtful cases the specimen was rejected. The identification was always performed by the same person. The material was collected in the course of several years from roughly the same area (Uusimaa Province). Several different techniques were used to measure the activity. The turbidimetric method proved the best for examining large numbers of samples. The transmission values given in Table 2 are the

means of two parallel determinations, and all the material shown in the table was examined in one working session in identical conditions. The values obtained were compared with earlier observations made by different techniques. If distinct differences emerged, the determination was re-checked. It was also possible to control the identification of the sample as the museum of the Institute of Botany, University of Helsinki has samples of the material listed in the table, especially of the more rare species. In case of doubt the sample was rejected.

The nomenclature of the material presented in Table 2 was based on the original works mentioned in the table. No synonyms have been used nor abbreviations of the author's names as they can be found in the works cited. The material was grouped into three systematic classes: Myxomycetes, or slime fungi; Ascomycetes, or sack fungi; and the greatest group, Basidiomycetes, or club fungi, the most characteristic structural feature of which is the basidium and which was further divided into subgroups. The possibility that the different parts of the fungus may vary in activity was controlled. No significant differences were found. No differences in activity were observed between fungi of different ages.

When air-dried fungus is crushed the extract of carelessly handled rough crushed fungus may be poorer in activity than an extract made of fine dust-like powder of the same species. An endeavour was made to avoid this mistake by crushing all the samples mechanically in doing which the air-dried fungus was pulverised into dust-like, very fine powder easy to extract and effective. No comparative quantitative examination was made of the amount of material extracted. On the other hand, the gradual decrease in activity was studied in some samples by means of several successive fractionating extracts. The first extract usually took up the bulk of the activity. The residual activity was so small that it was generally at the margin of detection by the methods used. In examination of the results shown in Table 2 account must be taken of the fact that the turbidimetric technique was calibrated to emphasise the differences between the enzyme activities in the fungus material. The reaction time was consequently kept as short as possible and if the sample studied revealed any enzyme activity it was

generally possible to obtain some idea of its relative strength compared with the other samples. A short reaction time reduces the other possibilities of error, for instance ever-present chance of spontaneous hydrolysis of the substrate.

The degrading activity for ribonucleic acid possessed by different fungus species does not follow the systematic order of the fungi. In other words, the fungi cannot be identified on the basis of their ribonucleic acid degrading effect. In some cases it would seem possible that some genera were more uniform than the family as a whole. In broad outline, the material examined seemed to show that the fungi known to be mycorrhizal generally had more distinct ribonucleic acid degrading effect than decaying fungi, especially fungi decaying wood. There were, however, numerous exceptions to this.

Assessment of enzyme activity according to the percentual hydrolysis of the substrate showed that the fungus species which degraded the substrate $\leqq$ 24 per cent could be considered practically inactive as the transmission readings ($T_{420} \leqq 19$—20) obtained were fairly close to the transmission value ($T_{420}=11$, Fig. 4) given by the substrate alone when precipitated. Activity in these cases must be regarded as at least doubtful. These species totalled 31, i.e. 13 per cent of the total material. Fungus species which degraded the substrate $\geqq$ 25 per cent could be regarded as slightly active. They totalled 63, i.e. 26 per cent of the material. Fungus species which degraded the substrate $\geqq$ 50 per cent could be regarded as fairly active. They totalled 65, i.e. 27 per cent of the material. Fungus species which degraded the substrate $\geqq$ 75 per cent could be considered as highly active. They totalled 83, i.e. 34 per cent of the material as a whole.

The above classification of the material by activity was entirely arbitrary. It shows that a distinct degrading activity for ribonucleic acid was possessed by 211 species, i.e. 87 per cent of the total material. This finding agrees with a previous study made by the present author with a smaller material and using a different technique (62).

CHARACTERISTICS OF THE FUNGUS ENZYMES ARMILLARIELLA MELLEA AND LACTARIUS TORMINOSUS

METHODS AND MATERIAL

Methods in general. — Two methods were used for measuring enzyme activity: The spectrophotometric method for the recording of increase in extinction at 260 mμ was employed to determine the optimal pH range and optimal temperature range, to ascertain the heat resistance of the enzymes and to calibrate the enzyme and substrate concentrations. Uranyl acetate perchloric acid, 1/5 of the volume of the reaction mixture, was used as the precipitant. The measurement was made from a specifically recorded suitable dilution of the filtrate. The measurements were generally made against a blank solution (buffer-enzyme-precipitant) and the extinction values expressed either as such or calculated against distilled water.

The turbidimetric reaction was employed solely in inhibition tests. The transmission values were determined within the framework of the standard method at 420 mμ after 20 min. had elapsed from precipitation. The samples were precipitated with an equal volume of 1 N HCl. All the measurements were carried out against blank solution (buffer-enzyme-precipitant).

Enzymes. — The fungus enzyme employed in the experiments reported in this section consisted of 1—2 per cent stock solutions of the lyophilisates of Armillariella mellea and Lactarius torminosus, made either in distilled water or the pertinent buffer. The working solutions were obtained by diluting the stock solution.

Crystalline pancreatic ribonuclease was used as a control. The stock solution of the enzyme was generally prepared by weighing 1 mg of enzyme into 100 ml of distilled water or the particular buffer used. The stability of the enzyme was ensured by adding to the stock solution 0.1 per cent gelatin. The

working solutions were made by diluting the stock solution (usually 1 : 20). The stock solutions were renewed as far as possible at short intervals to avoid errors due to weakened effectiveness. Crystalline pancreatic ribonuclease was used principally for the calibration of the method and to obtain parallel values for comparison with the fungus enzyme.

Substrate. — The preparation of stock solutions for the experiments of this section was highly individualised work and greatly dependent on the method applicable in each experiment. It is consequently impossible to give a uniform account of the preparation of the substrate stock solutions. The methods of preparation is reported in detail for each investigation object as the composition of the final reaction mixtures is described.

It was thought possible to employ commercial ribonucleic acid (mostly RNA, British Drug Houses, Ltd., London) in the tests in which increasing extinction at 260 mμ was used to follow enzyme hydrolysis. In general, however, an endeavour was made to use purified ribonucleic acid (RNA, Light & Co, Ltd, twice purified) the properties of which have already been described. Purified substrate only was used especially for the turbidimetric method since the variations in the acid solubility of the substrate may cause even great errors.

EXPERIMENTAL PROCEDURE AND RESULTS

EFFECT OF pH ON THE ACTIVITY OF THE ENZYMES

The variations in the solubility of ribonucleic acid in the different pH ranges and the resultant change in pH even in buffered solutions as well as the pH changes due to the addition of slightly acid fungus enzyme to the reaction mixture made it necessary always to use reaction mixture components prepared in the same manner and standardised carefully to a fixed volume.

Substrate stock solution I. — 62.5 mg of ribonucleic acid (British Drug Houses, Ltd., London) was dissolved in 10 ml of Michaelis buffer pH 9.55. The solution was made up immediately to the final volume of 25 ml with Michaelis buffer pH 2.45.

This 0.25 per cent stock solution was used in the preparation of the final reaction mixtures in the pH range 3.30—4.75.

Substrate stock solution II. — 62.5 mg of ribonucleic acid (British Drug Houses, Ltd., London) was dissolved in 25 ml of Michaelis buffer pH 9.55. This 0.25 per cent stock solution was employed in the preparation of the final reaction mixtures in the pH range 5.20—8.10.

Substrate-buffer solution. — The stock solutions described in the foregoing were diluted with distilled water, Michaelis buffers of various pH values and, on two occasions, by a small amount of 0.1 N HCl. This procedure gave 0.0625 per cent substrate-

TABLE 3

PREPARATION OF REACTION MIXTURES OF DIFFERING PH VALUES. SUBSTRATE I: 62.5 MG OF RNA (BRITISH DRUG HOUSES, LTD.) DISSOLVED IN 10 ML MICHAELIS BUFFER PH 9.55. ENOUGH MICHAELIS BUFFER PH 2.45 ADDED TO MAKE UP THE TOTAL VOLUME OF THE SOLUTION = 25 ML. SUBSTRATE II: 62.5 MG OF RNA (BRITISH DRUG HOUSES, LTD.) DISSOLVED IN 25 ML OF MICHAELIS BUFFER PH 9.55.

Substrate (0.25 %) ml		Distilled Water	0.1 N HCl	Michaelis Buffer, ml										Substrate-buffer Mixture (0.0625 %), pH	Addition of 1 % Fungus Enzyme, ml	Final pH of Reaction Mixture (RNA, 0.05 % + Fungus Enzyme, 0.2 %)
				pH Values												
I	II			2.45	3.20	3.65	4.20	4.72	5.32	7.40	7.95	8.55	9.55			
3		5.4	0.6	3										2.60	3	3.30
3		5.8	0.2	3										3.50	3	3.90
3		6			3									4.15	3	4.35
3		6						3						4.75	3	4.75
	3	6		3										5.15	3	5.20
	3	6				3								5.65	3	5.70
	3	6					3							6.30	3	6.15
	3	6						3						6.95	3	6.65
	3	6							3					7.40	3	7.10
	3	6								3				7.80	3	7.55
	3	6									3			7.95	3	7.70
	3	6										3		8.15	3	7.80
	3	6											3	8.45	3	8.10

buffer mixtures standardised to a fixed volume with pH values ranging from 2.60 to 8.45.

Final reaction mixture. — Adding a fixed volume of enzyme to the substrate-buffer solution gave a reaction mixture of the desired pH range and of a certain strength in regard to the substrate. The enzyme concentration was fixed for the reaction speed desired. The addition of crystalline ribonuclease to the substrate-buffer mixture no longer caused changes in the pH. This notwithstanding, the pH of the reaction mixture was always controlled. A distinct pH change, on the other hand, was produced by the addition of the 1 per cent fungus enzyme stock solution used. Hence the pH of the reaction mixture was always carefully checked at the moment the reaction started.

Table 3 shows in detail the method inclusive of the intermediate phases employed for the preparation of reaction mixtures of different pH values from various stock solutions. This procedure gave a substrate concentration of 0.05 per cent in all the reaction mixtures. The concentration of crystalline pancreatic ribonuclease in all the reaction mixtures was 0.00001 per cent. The concentration of the fungus enzymes used was 0.2 per cent in all the reaction mixtures.

Reaction rate in different pH ranges. — The following solutions pre-heated for 5 min. in a water bath at +37 °C in identical conditions were pipetted into the test tubes:

Reagent:		*Test:*	*Blank:*	*Control:*
Substrate:	RNA 0.625 mg/ml in different pH ranges	2 ml	—	2 ml
Enzyme:	(a) Crystalline PRNase 0.0005 mg/ml	0.5 „	0.5 ml	—
	(b) Armillariella mellea or Lactarius torminosus lyophilisate 10 mg/ml	0.5 „	0.5 „	—
Buffer:	Michaelis buffer corresponding to the final pH of the reaction mixture	—	2.0 „	0.5 „

The final substrate concentration of the 2.5 ml reaction mixture was thus 0.05 per cent, the final crystalline enzyme concentration 0.00001 per cent and that of the fungus enzyme 0.2 per cent in the different pH ranges. Uranyl acetate perchloric acid precipitant reagent was used to stop the effect of crystalline

ribonuclease on the reaction mixture 10 min. and that of the fungus enzyme 25 min. from the beginning of the reaction by adding 1/5 volume of the precipitant. The solutions were filtered 10 min. after precipitation and extinction was determined at 260 mμ from the 0.3 : 5.0 buffer dilution of the filtrates 30 min. after the reaction was stopped. The extinctions were measured against a blank solution (buffer-enzyme-precipitant) treated in the same way. Spontaneous hydrolysis of the substrate was followed from the control reaction mixture which contained no enzyme and was precipitated, filtered and diluted in the same way as the actual reaction mixture. The final extinction values were calculated against distilled water. The reliability of the method is seen in the fact that with crystalline pancreatic ribonuclease the optimal pH was measured very close to its known correct pH value 7.7.

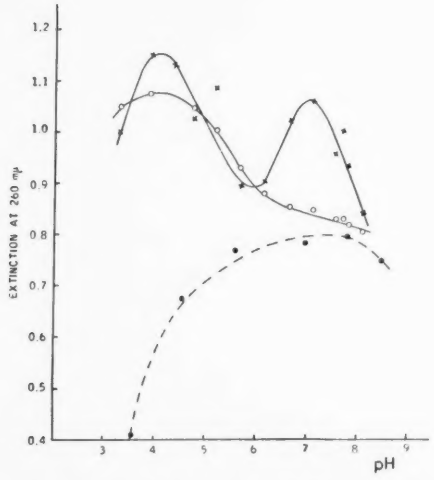

Fig. 5. — The effect of pH on the ribonucleolytic activity of fungus enzymes. Reaction mixture: RNA (British Drug Houses, Ltd., London) 0.625 mg/ml, 2.0 ml. Lyophilised fungus enzyme 10 mg/ml, 0.5 ml. The pH of the reaction mixture was adjusted more accurately to the desired value, generally with Michaelis buffer according to Table 3. Reaction temperature +37 C. Reaction time 25 min. 0.5 ml of crystalline pancreatic ribonuclease 0.0005 mg/ml was added to the same substrate. The reaction conditions were identical except that the reaction time was 10 min.

×——×——× Armillariella mellea fungus enzyme
o——o——o Lactarius torminosus fungus enzyme
●– –●– –● Crystalline pancreatic ribonuclease

Fig. 5 gives the results of the effect of pH on the activity of Armillariella mellea and Lactarius torminosus. The extinction values recorded are means of six parallel determinations measured separately at each pH. The figure also shows the behaviour of crystalline pancreatic ribonuclease in identical test conditions. The optimal pH of both Armillariella mellea and Lactarius torminosus fungus enzymes was 4.0. Armillariella mellea fungus enzyme had another pH optimum, 7.1, although activity there was less than at the acid pH optimum. The Lactarius torminosus fungus enzyme showed no increase in activity in the neutral and alkaline pH range. With Armillariella mellea the reaction was more difficult to control at pH 7.1, and this accounts for the greater deviations in the results. Hence the fungus enzymes differed from one another in their pH optima. For crystalline pancreatic ribonuclease the optimal pH was 7.7.

HEAT STABILITY OF FUNGUS ENZYMES

Prior to the optimal temperature studies, the inactivating effect of higher temperatures on the activity of fungus enzymes was determined. The heat resistance of fungus enzymes was examined at pH 4 only since the reaction was difficult to control at pH 7.1. Lyophilisates of Armillariella mellea and Lactarius torminosus fungus enzymes were used for the tests. The spectrophotometric method was employed to record the enzymatic effect and the same method served to determine the conditions for the preparation of the reaction mixture.

Acid reaction mixture (Table 3) was made for use with the spectrophotometric method which registers the increase in extinction at 260 mμ. The final pH of the reaction mixture was 3.9 and its substrate concentration 0.05 per cent. Only the concentrations of the enzyme in the reaction mixture and the heat treatments differed from those shown in Table 3.

Effect of heat on the activity of fungus enzymes. — +100 °C for 10 min.: The effectiveness of boiled fungus enzymes was examined by a method recording the increase in extinction at 260 mμ. 10 ml of 1 per cent Armillariella mellea and Lactarius torminous fungus enzyme lyophilisate was boiled for 10 min. in a

water bath at +100 °C before use in the reaction mixture. The solutions evaporated to about half their volume during the boiling and a part of the enzyme proteins was precipitated. The cooled solution was made up with the same solvents to its original volume and the solution thus obtained was employed as the enzyme component in the reaction mixture in the usual manner. Samples taken at suitable time intervals from the reaction mixture were precipitated by adding uranyl acetate perchloric acid reagent 1/5 of the sample volume. The increase in extinction at 260 mμ was followed from the 0.5 : 5.0 buffer dilutions of the filtrates. No change in extinction, however, was observed in the course of the reaction time of 1 hour. This means that

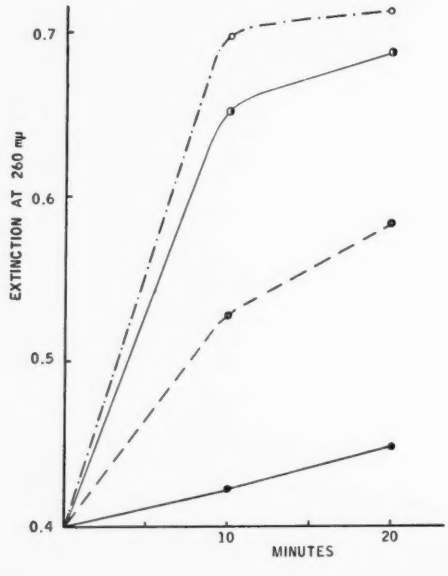

Fig. 6. — The effect of heat treatment on the ribonucleolytic activity of fungus enzymes. Reaction mixture: RNA (British Drug Houses Ltd., London) 0.625 mg/ml, 2.0 ml. Armillariella mellea and Lactarius torminosus lyophilisate 10 mg/ml, 0.5 ml. The enzyme solutions were heated at +60 °C for 1 hour. The pH of the reaction mixture was adjusted according to Table 3 to pH 3.9 with Michaelis buffer. Reaction temperature +37 °C. Reaction time 20 min.

Armillariella mellea, unheated
Armillariella mellea, heated for 1 hour at +60 °C
Lactarius torminosus, unheated
Lactarius torminosus, heated for 1 hour at +60 °C

both enzymes were completely inactivated by 10 minutes boiling.

+70 °C for 1 hour: The activity of the same fungus enzymes after +70 ° C heat treatment was studied by the same method. Evaporation losses were made good in the same way. The Lactarius torminosus fungus enzyme was inactivated completely at this temperature too, whereas the Armillariella mellea fungus enzyme retained a weak enzyme activity (c. 10 per cent of the original activity).

+60 °C for 1 hour: The activity of the same fungus enzymes after +60 ° C heat treatment was studied by the same method as in the preceding tests. Fig. 6 shows the results as extinction changes measured at 260 mμ in comparison with the behaviour of the same enzymes not heat treated. The substrate concentration in the reaction mixture was 0.05 per cent and the enzyme concentration 0.2 per cent. The more heat-labile Lactarius torminosus fungus enzyme was still largely inactivated at this temperature. C. 15 per cent remained of the original enzyme activity. The Armillariella mellea fungus enzyme, on the other hand, withstood +60 ° C heat treatment well and c. 60 per cent of the original enzyme activity remained. Heat stability thus seemed to differ distinctly in the two fungus enzymes studied.

EFFECT OF TEMPERATURE ON THE ACTIVITY OF FUNGUS ENZYMES

The effect of temperature on the reaction velocity was studied with Armillariella mellea fungus enzyme at both its pH optima and with Lactarius torminosus fungus enzyme at pH 3.9 only.

Acid reaction mixture. — The substrate-buffer mixture was prepared exactly as shown in Table 3. After the addition of enzyme the final pH of the reaction mixture was 3.9 and its substrate concentration was 0.05 per cent. The enzyme concentration was 0.2 per cent.

Neutral reaction mixture. — The substrate-buffer mixture and the final reaction mixture were made as shown in Table 3. The final pH of the reaction mixture was 7.1, its substrate concentration 0.05 per cent and the enzyme concentration was 0.2 per cent.

Determination of the optimal temperature for fungus enzyme effect. — The method employed was the increase in extinction at 260 mμ caused by acid-soluble degradation products. The experiments were made in a thermostat in which the temperature could be adjusted with great accuracy. The following temperatures were used: +6 °C (in a refrigerator), +20 °C, +40 °C, +50 °C, +60 °C and +80 °C. The reaction mixture components were pre—heated for 15 min. at temperatures corresponding to the test conditions, then pipetted into conical flasks in the following manner:

Reagent:		*Test:*	*Blank:*	*Control:*
Substrate:	RNA 0.625 mg/ml, pH 3.5 or 7.4	20 ml	—	20 ml
Enzyme:	Armillariella mellea or Lactarius torminosus lyophilisate 10 mg/ml	5 ,,	5 ml	—
Buffer:	Michaelis buffer corresponding to the final pH of the reaction mixture	—	20 ,,	5 ,,

The final substrate concentration of 25 ml of reaction mixture was 0.05 per cent and the final concentration of the fungus enzyme 0.2 per cent. The final pH of the reaction mixture was adjusted to pH 3.9 and pH 7.1. Two samples of 2 ml each were taken from the reaction mixture at the 0 moment and, during incubation, after 10, 20, 40 and 60 min. The samples were precipitated by adding 1/5 volume of uranyl acetate perchloric acid reagent. Extintion measurements were made at 260 mμ against a blank solution (buffer-enzyme-precipitant) from the 0.3 : 5.0 buffer dilutions of the filtrates. For the final explanation the means of the extinctions of parallel determinations were calculated against distilled water in order to eliminate the potential error of different test series. The spontaneous hydrolysis of the substrate was controlled from a reaction mixture without enzyme. No spontaneous hydrolysis was seen. The errors due to evaporation loss in tests performed at +80 °C were eliminated by calculating a coefficient of correction for the incubation times used. For this purpose, evaporation (1.56 g) from 25 ml in 1 hour at +80 °C in the reaction vessel used was first determined experimentally.

Figs. 7 and 8 give the results of the tests made at both pH optima. The extinction values shown are the means of two similar experiments, i.e. total of four parallel determinations

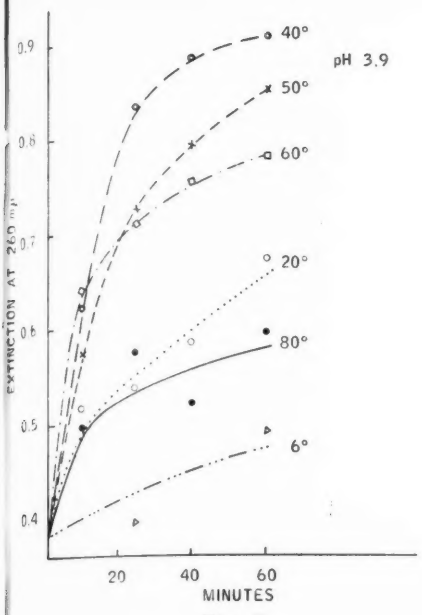

Fig. 7.

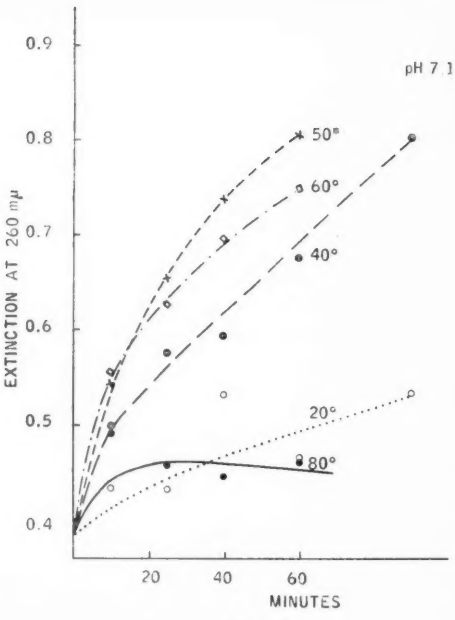

Fig. 8.

Figs. 7 and 8. — The effect of temperature on the ribonucleolytic activity of Armillariella mellea measured at both its pH optima.
Reaction mixture: RNA (British Drug Houses, Ltd., London) 0.625 mg/ml, 20 ml. Armillariella mellea or Lactarius torminosus lyophilisate 10 mg/ml, 5 ml. The pH values of the reaction mixture were adjusted according to Table 3 to pH 3.9 and 7.1 with Michaelis buffer. Reaction time 60 min.

from samples taken at the measuring points of each temperature studied. It will be seen from Figs. 7 and 8 that the optimal temperature at pH 3.9 for the fungus enzyme activity of Armillariella mellea was +40 °C. At pH 7.1 the optimal temperature for the same fungus enzyme in identical conditions was +50 °C. The optimal temperature at pH 3.9 for the Lactarius torminosus fungus enzyme was likewise +40 °C. This temperature is not stated separately in Fig. 7. To summarise, the fungus enzymes of Armillariella mellea and Lactarius torminosus showed no difference in optimal temperatures at the pH optimum common to both of them. On the other hand, distinctly different temperature optima were obtained for the different pH optima of Armillariella mellea fungus enzyme.

EFFECT OF THE CONCENTRATION OF FUNGUS ENZYME ON THE REACTION VELOCITY

The effect of the changes in the enzyme concentration on the reaction rate was studied with Armillariella mellea only. The method employed was measurement of the increase in extinction at 260 mμ which determined the principles applied in the preparation of the reaction mixtures.

Acid reaction mixture, the final pH of which was 4.0 and the final substrate concentration 0.05 per cent, was prepared as shown in Table 3. The only variation was the different concentrations of Armillariella mellea used in the reaction mixture. They were obtained by adding to 2 ml of 0.0625 per cent substrate 0.5 ml of 2 per cent fungus enzyme stock solution or a suitable dilution to vary the enzyme concentration in the final reaction mixture from 0.4 per cent to 0.025 per cent.

Neutral reaction mixture, final pH 7.1 and final substrate concentration 0.05 per cent, was prepared according to Table 3. The enzyme concentrations in the reaction mixture was varied as in the foregoing.

Effect of various enzyme concentrations on the course of the reaction with the substrate concentration remaining constant was studied at pH 4.0 and 7.1 by recording the increase in extinction at 260 mμ. Reaction mixture components pre-heated in a water bath at +37 °C for 10 min. in the same conditions and according to the previously described formula were pipetted into the test tubes. The final concentration of substrate was kept carefully constant at 0.05 per cent in the reaction mixture. The enzyme concentrations ranged from 0.4 per cent to 0.025 per cent. After 10 min. of digestion the reaction was stopped by precipitating a 2 ml sample with 1/5 volume of uranyl acetate perchloric acid reagent. The extinctions were measured from 0.2 : 5.0 buffer dilutions of the filtrates against the corresponding blank solution. Spontaneous hydrolysis of the substrate was observed in a control reaction containing no enzyme. The final extinction values, obtained from the means of the parallel determinations, were calculated against distilled water.

Fig. 9 shows the results of the effect of changes in the concentration of Armillariella mellea fungus enzyme on the reaction rate when the substrate concentration was kept constant. The

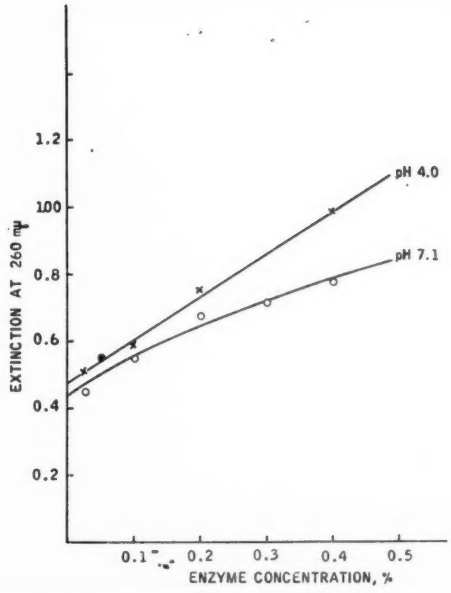

Fig. 9. — The effect of Armillariella mellea fungus enzyme concentration on the reaction rate. Reaction mixture: RNA (British Drug Houses, Ltd., London) 0.625 mg/ml, 2.0 ml. Armillariella mellea lyophilisate in different concentrations, 0.5 ml. The final pH of the reaction mixture was adjusted with Michaelis buffers to both the pH optima, 4.0 and 7.1, according to the Table 3. Reaction temperature 37 °C. Reaction time 10 min.

×——×——× Reaction rate at pH 4.0
o——o——o Reaction rate at pH 7.1

course of the reaction was linear at both the pH optima of the! Armillariella mellea enzyme in the enzyme and substrate concentration range studied. Thus, the limiting velocity attained in the presence of an excess of substrate is proportional to the concentration of enzyme, which is in good agreement with the theory proposed by Michaelis and Menten (79).

EFFECT OF SUBSTRATE CONCENTRATION ON REACTION VELOCITY

Although the method used involved following the increase in extinction at 260 mμ, it was not possible to make the reaction mixtures according to Table 3. The aim here was to prepare reaction mixtures of different substrate concentrations but constant enzyme concentration.

Acid substrate stock solution. — 250 mg of ribonucleic acid (RNA, British Drug Houses, Ltd., London) was dissolved in 25 ml of the most basic Michaelis buffer pH 9.55. After complete dissolution the pH dropped to c. 6. 10 ml of 0.1 N HCl and distilled water *ad* 50 ml were added. The final pH of the stock solution was 3.65 and its substrate concentration 0.5 per cent. Suitable buffer dilutions of this stock solution were used to prepare reaction mixtures of different substrate concentrations. The final pH of these reaction mixtures was 4.0 and the enzyme concentration a constant 0.2 per cent.

Alkaline substrate stock solution. — 250 mg of ribonucleic acid (RNA, British Drug Houses, Ltd., London) was dissolved in 25 ml of the most basic Michaelis buffer pH 9.55. 0.5 ml of 1 N HCl and distilled water *ad* 50 ml were added. The final pH of the stock solution was 7.45 and its substrate concentration 0.5 per cent. Suitable buffer dilutions of this stock solution were used to prepare reaction mixtures of different substrate concentrations. The final pH of these reaction mixtures was 7.1 and the enzyme concentration a constant 0.2 per cent.

Final reaction mixture. — The preparation procedure was as follows: both 0.5 per cent substrate solutions were diluted 1 : 2, 1 : 4, 1 : 8, 1 : 16 and 1: 32 with the corresponding Michaelis buffer so that the pH did not change. 2 ml of each dilution mixed with 0.5 ml of 1 per cent of Armillariella mellea solution gave a 0.2 per cent enzyme concentration. The substrate concentrations ranged from 0.1 per cent to 0.0125 per cent. The final pH of the reaction mixtures were 4.0 and 7.1.

Effect of various substrate concentrations on the course of the reaction when the enzyme concentration remains constant was studied at pH 4.0 and 7.1 by recording the increase in extinction at 260 mμ. Reaction mixture components pre-heated in a water bath at +37 °C for 10 min. in the conditions of the test procedure described previously were pipetted into test tubes. 0.5 ml of enzyme with various controls was pipetted into 2 ml of each substrate dilution. After a reaction time of 5 min. each tube was diluted prior to precipitation with distilled water so that the substrate concentration was the same in each tube, i.e. 0.0125 per cent. The reaction was then stopped by pipetting rapidly 1/5 volume of uranyl acetate perchloric acid.

The extinctions were measured at 260 mμ from 0.5 : 5.0 aqueous dilutions of the filtrates against distilled water 30 min. after stopping the reaction. 5—10 determinations were made in identical test conditions for each substrate concentration used.

The reaction was extremely difficult to control and the total extinction values tended to vary accordingly. Acceptable and mutually comparable results were obtained only from measurements in which the reaction mixtures were diluted in the same substrate concentration prior to precipitation. It was difficult to express the results in total extinction values and they were consequently expressed as extinction differences (Δ E) occurring during the reaction time in question. These values were obtained by deducting from the mean total extinction values the sum

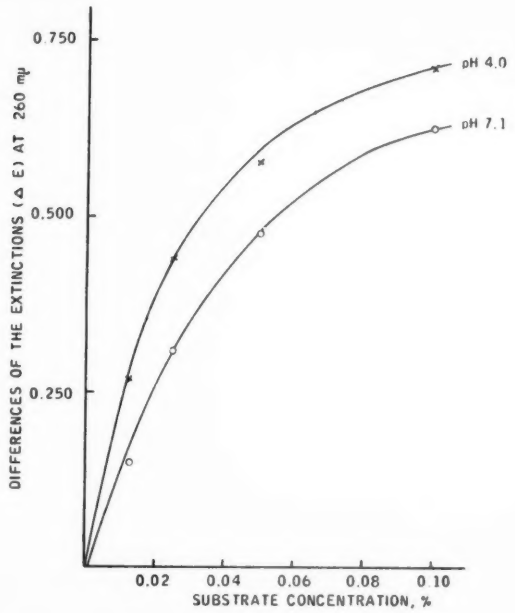

Fig. 10. — The effect of substrate concentration on the reaction velocity. Reaction mixture: RNA (British Drug Houses, Ltd., London) in different concentrations, 2.0 ml. Armillariella mellea lyophilisate 10 mg/ml, 0.5 ml. The final pH of the reaction mixtures were adjusted to both the pH optima of the enzyme, 4.0 and 7.1, with Michaelis buffers. Reaction temperature +37 °C. Reaction time 5 min.

×——×——× Reaction rate at pH 4.0
o——o——o Reaction rate at pH 7.1

of the blank value (buffer-enzyme-precipitant) corresponding to the dilution of each reaction mixture and the 0-control of the substrate (amount of RNA in the reaction mixture).

Fig. 10 shows the effect of different substrate concentrations on the reaction rate when the concentration of Armillariella mellea fungus enzyme was constant. The reaction velocity at both pH optima of the fungus enzyme was plotted as a function of the different substrate concentrations. The curve obtained had the shape of a rectangular hyperbola. Thus, using a fixed quantity of enzyme the initial reaction velocity increases with increasing substrate concentration up to a certain limit. The limit velocity finally attained depends upon and is in fact proportional to the enzyme concentration. These experimental observations are also in agreement with the Michaelis-Menten theory (79).

INHIBITION OF ENZYME ACTIVITY

Only Lactarius torminosus fungus enzyme in lyophilised form was used for the inhibition experiments. The inhibitors selected were generally recognised cations, some of them known to have an inhibitory effect on crystalline pancreatic ribonuclease. The turbidimetric reaction was the only investigation method employed.

Concentration of the inhibitors in the reaction mixture was determined in preliminary tests and the inhibitors were accordingly classified into groups. In the lowest concentration of each group the inhibition effect was at the margin of observability. In the highest concentration inhibition was distinctly observable without concurrent precipitation of the substrate with the reaction mixture. If the inhibitor concentration in the reaction mixture was increased further the consequence was generally partial or complete precipitation of the substrate.

The inhibitors examined were grouped in the following concentration classes:

A. 2×10^{-4}M, 2×10^{-3}M, 4×10^{-3}M.
Copper acetate, cobalt chloride, manganese sulphate and zinc sulphate.

B. 1×10^{-1}M, 2.5×10^{-1}M, 5×10^{-1}M.
ammonium sulphate, calcium chloride, magnesium sulphate and sodium chloride.

C. 10 mg%, 50 mg%, 100 mg%.
Atebrin, Heparin, Penicillin G and Dihydrostreptomycin.

As transmission measurements at 420 mμ were employed as the investigation method the reaction mixture was calibrated to a final substrate concentration of 0.05 per cent in the reaction mixture and enzyme concentration of 0.1 per cent. The pH was 4.0. The total volume of reaction mixture used was 5 ml, obtained by adding 2 ml of 0.125 per cent substrate to 1 ml of the inhibitor solution. 2 ml of 0.25 per cent enzyme was added finally. The inhibitor had to be added to the substrate in a concentration 5 times higher than the intended final inhibitor concentration of the reaction mixture. This did not cause precipitation of the substrate.

Ion concentration of the buffer in the reaction mixture. — As far as possible, the buffer accounted for 2/5 of the volume in all the reaction mixtures. The buffer in most frequent use was Michaelis isotonic buffer series of wide pH range. To obtain a pH of 4.0 in the reaction mixture, the substrate was always dissolved either direct in the buffer as such or in a suitable aqueous dilution of the buffer. For reasons of caution, however, the buffer was not used in its original form as its ion concentration is 0.17 M. As a 2/5 dilution in the final reaction mixture the ion concentration produced by the buffer would have been 0.069 M. Hence NaCl was omitted altogether from the buffer because of the inhibition risk. The ion concentration of the thus modified buffer as a 2/5 dilution in the final reaction mixture was only 1/3 of the former, i.e. 0.023. The buffer was found to have no inhibitory effect in this concentration on the course of the reaction. This was controlled by comparing the reaction velocity in the same conditions with parallel determinations at pH 4.0 in acetate buffers of different ion concentrations.

Measurement of inhibitory effect. — The inhibitory effect of different substances was examined in the reaction mixture range described above by means of turbidity reaction at 420 mμ. The reaction was sufficiently rapid in these conditions and the technique sensitive enough to record even small inhibitory ef-

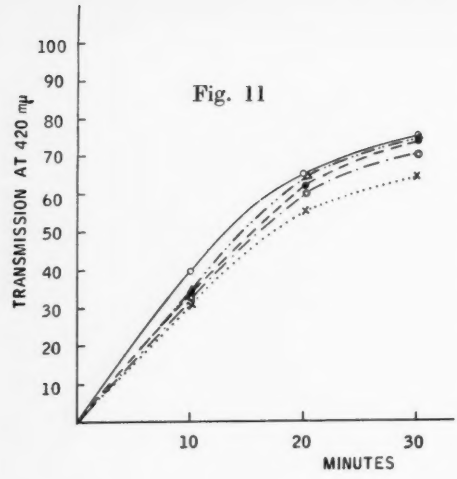

Fig. 11.— The inhibitory effect of various cations on the ribonucleolytic activity of Lactarius torminosus. Reaction mixture: RNA (Light & Co, Ltd., twice purified) 1.25 mg/ml, 2.0 ml. Enzyme 2.5 mg/ml, 2.0 ml. The inhibiting agent in 1.0 ml had a concentration which was equivalent to a final con- centration of 2×10^{-4}M in the reaction mixture. The final pH of the reaction mixture was 4.0. Reaction time 30 min. Reaction temperature + 37 °C

- Normal reaction
- $CoCl_2$
- $ZnSO_4$
- $MnCl_2$
- $Cu(CH_3COO)_2 + H_2O$

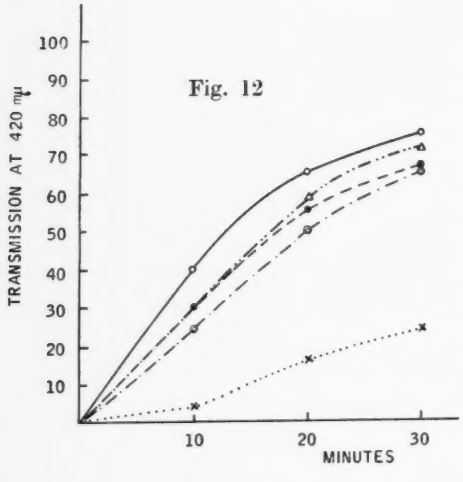

Fig. 12. — The inhibitory effect of various cations on the ribonucleolytic activity of Lactarius torminosus. The reaction mixture was the same as in the experiments presented in Fig. 11 with the exception that the concentration of the same inhibitors in the final reaction mixture was 2×10^{-3}M.

- Normal reaction
- $CoCl_2$
- $ZnSO_4$
- $MnCl_2$
- $Cu(CH_3COO)_2 + H_2O$

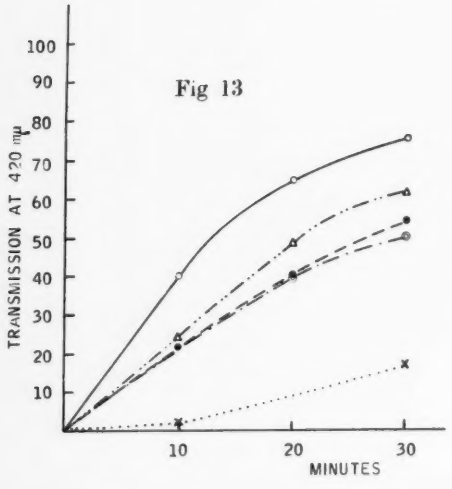

Fig. 13. — The inhibitory effect of various cations on the ribonucleolytic activity of Lactarius torminosus. The reaction mixture was the same as in the experiments presented in Fig.11 with the exception that the concentration of the same inhibitors in the final reaction mixture was 4×10^{-3} M.

- Normal reaction
- $CoCl_2$
- $ZnSO_4$
- $MnCl_2$
- $Cu(CH_3COO)_2 + H_2O$

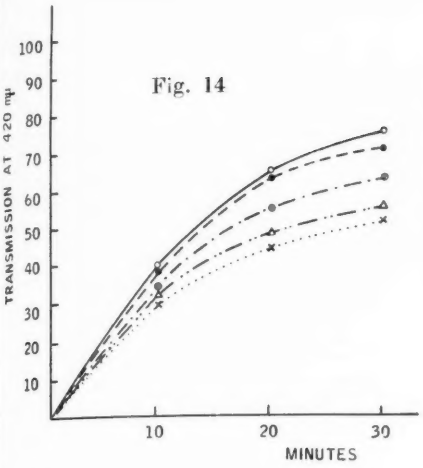

Fig. 14. — The inhibitory effect of various cations on the ribonucleolytic activity of Lactarius torminosus. The reaction mixture was the same as in the experiments presented in Fig. 11 with the exception that the concentration of the inhibitors in the reaction mixture was 2.5×10^{-1}M.

Normal reaction
$(NH_4)_2SO_4$
$CaCl_2$
NaCl
$MgSO_4$

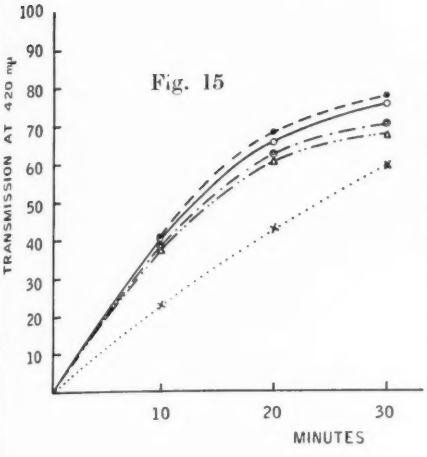

Fig. 15. — The effect of penicillin, atebrin, heparin and dihydrostreptomycin on the ribonucleolytic activity of Lactarius torminosus. The final reaction mixture concentration was 100 mg %. The reaction mixture was otherwise the same as in the experiments presented in Fig. 11.

Normal reaction
Penicillin
Atebrin
Heparin
Dihydrostreptomycin

fects. Reaction mixture components pre-heated for 10 min. in a water bath at +37 °C in the same conditions were pipetted into test tubes in the following order:

Reagent:		*Test:*	*Blank:*	*Control:*
Substrate:	RNA 1.25 mg/ml, pH 3.85	2 ml	—	2 ml
Inhibitor:	Concentration 5 times that reported above	1 ,,	1 ml	1 ,,
Enzyme:	Lactarius torminosus 2.5 mg/ml	2 ,,	2 ,,	—
Buffer:	Michaelis buffer pH 4.0	—	2 ,,	2 ,,

The final concentration in the reaction mixture was 0.05 per cent for the substrate and 0.1 per cent for the enzyme. The final pH of the reaction mixture was 4.0. The hydrolysis was followed at 10, 20 and 30 minute intervals by precipitating a 1 ml sample with the corresponding amount of 1 N HCl. The transmission measurements were made 20 min. after precipitation at 420 mμ against the pertinent blank solution (buffer-enzyme-inhibitor-precipitant). The control in each test series was the corresponding reaction mixture without inhibitor and another reaction mixture without enzyme. The controls were used to follow the course of uninhibited reaction. No spontaneous hydrolysis of the substrate was seen. Special attention was paid to possible precipitation of the substrate in the presence of an inhibitor.

Figs. 11, 12, 13, 14 and 15 show the results of the inhibition studies.

The order of the cations examined in the concentrations 4×10^{-3}M, according to their inhibitory ability, was as follows:

$$Cu^{++} > Mn^{++} > Zn^{++} > Co^{++}$$

In the concentrations 2.5×10^{-1}M the order of the other cations examined, by their inhibitory ability, was:

$$Mg^{++} > Na^{+} > Ca^{++} > NH_4^{+}$$

In the concentrations 100 mg% the order of the other substances examined, by their inhibitory ability, was:

Dihydrostreptomycin > Heparin > Atebrin

Penicillin did not inhibit the reaction in any of the concentrations studied. By contrast, in the concentration 100 mg%, it seemed to activate the reaction slightly judging by the turbidity reaction. It was impossible, however, to demonstrate activation clearly when controlled by several different techniques.

No high-efficiency inhibitor was found among the substances examined in the present investigation. Copper, which exerted the most pronounced inhibitory effect on the reaction, also had the greatest tendency to precipitate the substrate in the reaction mixture. Nor did any of the agents examined activate the process.

DISCUSSION

The choice of methods for study of the optimal conditions for the effect of fungus enzyme was governed primarily by the object of the investigation.

Optimum pH determinations were made by recording the increase in extinction at 260 mμ caused by acid-soluble degradation products. In the acid pH range the decrease in extinction at 300 mμ could not be used for recording pH effect since the decrease in extinction at this wavelength is confined to a very limited pH range near 5.0. The turbidimetric technique was also used to study the effect of pH on the reaction rate, but the technique proved too inflexible. This method, too, gave a distinctly bi-phasic pH curve for Armillariella mellea, indicating the presence of two different pH optima. With the Lactarius torminosus fungus enzyme the pH optimum curve was monophasic.

Recording the increase of acid-soluble degradation products in the reaction mixture proved likewise to be the best method of determining the optimal temperature. In view of the very many potential errors numerous parallel determinations were made in several identical test series. Of the factors of error may be mentioned the spontaneous hydrolysis of the substrate at higher temperatures. When this was observed the entire test series was interrupted and the results rejected. Evaporation, also, at higher temperatures causes a decrease in the volume of the reaction mixture thus introducing, especially in the last samples, errors due to changes in the concentrations. This was taken into consideration in the experiments made at +80 °C in which coefficients of error were calculated for the incubation times used and the remaining reaction mixture volumes. The thermal expansion of volume reduces the concentrations in the reaction mixture but this was not followed in the determinations as the increase in the specific volume of water between +20 °C and +80 °C is only c. 2.6 per cent.

In studying the heat resistance of fungus enzymes a comparison was made between the spectrophotometric method employed in the previous tests and the turbidimetric technique. The latter method was more difficult to calibrate when using heat-treated

preparations and preparations of differing enzyme activity. Moreover, when correcting for evaporation losses it was sometimes difficult to make enzyme stock solutions, treated at higher temperatures and partly precipitated and denatured, re-dissolve. Such a reaction mixture component cannot be used in the turbidimetric method. It was therefore decided to measure acid soluble degradation products at 260 mμ.

The measuring of acid soluble degradation products at 260 mμ was the sole method used to study the effect of various enzyme and substrate concentrations on the course of the reaction. With this technique it was easy to control the reaction when the enzyme concentrations were changed. On the other hand, when different substrate concentrations were used, it was very difficult to follow the course of the reaction primarily because of the measuring bases. It appeared that the acid solubility of the substrate was clearly dependent on its concentration: the acid solubility of the substrate increased the more it was diluted. This was observed even when the dilution was carried out simultaneously with the addition of the precipitant. The errors arising from changes in the acid solubility of the substrate were eliminated by diluting all the reaction mixtures to the same substrate concentration prior to precipitation. The precipitations were made from the dilutions of the reaction mixture in which the final concentration of the substrate was 0.0125 per cent. Extinction measurements were made from 0.5 : 5.0 dilutions of the filtrates against distilled water. Correct assessment of the blank values (buffer-enzyme-precipitant) was important for the calculation of final results. When the blank solutions were diluted in different ways against the substrate concentrations the measuring results varied greatly. Besides, it is not impossible that the precipitant which is in itself a strong oxidising agent reacts very differently with enzyme solutions of different strengths. This question has not been fully clarified. To obtain reliable results each blank solution was prepared separately for each substrate concentration of the reaction mixture to be precipitated or for its final measuring dilution. In the final analysis of the results the mean of a total of 40 blank determinations was compared with the measuring result of each individual reaction mixture component. Moreover, when the same precipi-

tant volumes were employed in the precipitation and the same dilutions used in the extinction measurements, the variation of the blank values was succesfully kept within reasonable limits. The final results of the substrate concentration studies were developed by deducting the sum of the blank extinction corresponding to the dilution of the reaction mixture and of the 0-control of the substrate from the mean extinction of the parallel determination diluted in the same substrate concentration. The difference was then corrected for the same dilution. The results illustrate only the extinction differences (Δ E) in the reaction time used, not the increase in extinction.

The turbidimetric technique was applied in measuring the inhibitors. The errors araising from the different acid solubilities were avoided by using a purified substrate with well known properties. This procedure gave even, reliable readings and there were hardly any variations in the normal reaction of the different experimental series. The technique was made sufficiently sensitive by suitably calibrating the enzyme concentration. Hence it was possible by comparing the course of the reaction with the standard reaction to record even a small deviation caused by the inhibitors. The only drawback was the substrate-precipitating effect of some of the cations above certain concentrations. However, these cations were found to have a distinct inhibitory effect on the reaction already in considerably lower concentrations and with this known it was possible to carry out the investigations without any disturbances.

In assessing the results obtained special attention is attracted first and foremost by the different behaviour of the fungus enzymes examined when determining the optimal conditions for their function.

Armillariella mellea fungus enzyme had a bi-phasic pH curve in pH optimum determinations. For the Lactarius torminosus fungus enzyme there was a mono-phasic curve and it revealed the same acid pH optimum as Armillariella mellea. The activity of Armillariella mellea fell sharply immediately outside the pH optima, whereas Lactarius torminosus was active over a much wider pH range on both sides of the optimum point. This, in determining enzyme activities at pH 5.0, produces a certain error for Armillariella mellea. Moreover, as Armillariella mellea

deviated most on both sides of pH 5.0, calculation of the coefficient of correction for activity loss measured at an incorrect pH is a very uncertain proposition and no such attempt was made. In assessing the reliability of the results it will be well to remember the differing inhibition in the different pH ranges by the metals present in the substrate as impurities (43). Such metals are e.g. Ca and Fe, and their inhibitory effect grows with the alkalinity of the reaction mixture. Location of the optimum pH is thus dependent on the metal inhibitors present in the substrate as impurities. The substrate used in the present investigation was not analysed in this respect. However, crystalline pancreatic ribonuclease should involve the greatest potential error as its optimum activity is in the alkaline pH range. The correct value, pH 7.7, was nevertheless obtained for the optimum pH of crystalline pancreatic ribonuclease, which indicates that this source of error was of no great significance here.

The difference of both the fungus enzymes studied were emphasised further by the thermostability examinations. The Armillariella mellea fungus enzyme was relatively heat-stable. Lactarius torminosus, a more thermolabile enzyme, was easy to destroy by suitable heat treatment.

The optimal temperature, on the other hand, was the same for both fungus enzymes at the acid pH optimum. The optimal temperature of Armillariella mellea at the neutral pH optimum differed however, from the optimal temperature of the acid pH optimum of the same fungus enzyme. The existence of two pH optima for the same enzyme and the corresponding different optimal temperatures seemed also to be correlated with the different reaction velocities of the enzyme at the two pH optima. According to preliminary observations, the reaction rate was slower at the neutral pH optimum than at the acid pH optimum, although the enzyme concentration was kept strictly constant. These observations may perhaps indicate the presence in the same fungus of two enzymes differing from one another and nevertheless similar in effect. Because of this, only Armillariella mellea was used to study the effect of the enzyme and substrate concentrations on the reaction rate, the object being to obtain additional evidence for the above assumption.

In the more detailed examination the reaction velocity indeed proved to be different at the two pH optima of the Armillariella mellea enzyme in the substrate concentrations examined. The reaction rate was clearly higher at the acid pH optima than at the neutral pH optimum. The effect of the different concentrations of the enzyme and the substrate on the reaction velocity appeared moreover to be in conformity with the theory of Michaelis-Menten. Thus, with the presence of an excess of substrate the limiting velocity attained was proportional to the concentration of enzyme, and with a fixed quantity of enzyme the initial reaction velocity increased with inereasing substrate concentration up to a limiting value. The magnitude of the limiting velocity finally attained depended upon the enzyme concentration used and was, in fact, proportional to that concentration. Hence, the shape of the curve plotted was a rectangular hyperbola.

Only the Lactarius torminosus fungus enzyme, at pH 4.0, was used for inhibition studies, as it had previously appeared to be more readily affected by external factors. The inhibition studies were not conducted systematically. The purpose here was merely to examine the effect of some ionised substances, especially metal salts, on fungus enzyme activity since these ions are the most common disturbing factors (they usually occur as buffer components in the reaction milieu and may be present as impurities in the substrate). The results of the inhibition experiments showed immediately that no specific inhibitor was found among the substances investigated. Cu^{++} inhibited the reaction most, but even it did not do so except in relatively high concentrations. Its effect was probably exerted on the substrate rather than on the enzyme as it had a clear substrate precipitating influence in the higher concentrations used. The inhibitory effect of the other substances studied was slight. The inhibitory effects observed were taken to be cation effects. Acetate effect was excluded in acetate buffer experiments in which no inhibitory effect was observed as compared with Michaelis buffer prepared without adding NaCl. As the reaction-inhibiting influence of $CaCl_2$ was small in a very high concentration (Fig. 14), the anion effect in a $CoCl_2$-concentration of an altogether different magnitude could not have played any role. The same obvious applied to SO_4^{--}

As both the examined fungus enzymes, Armillariella mellea and Lactarius torminosus, appeared to differ from one another in many respects, it seemed natural to assume that their active enzyme components were of differing character although similar in effect. More complete and especially comparative studies of a material consisting of several different fungus enzymes might clarify the problem further.

DEGRADATION PRODUCTS IN THE DIGESTION OF RIBONUCLEIC ACID BY THE TWO FUNGUS ENZYMES

METHODS AND MATERIAL

Methods in general. — For phosphorus determinations in making simple inorganic phosphorus analyses, use was made of the microtechnique developed by Berenblum and Chain (8). This technique was reported on in detail earlier. To measure radioactive inorganic phosphorus the method used was that reported by Roth and Milstein (89). This involves calibration of concentrations to correspond to the radioactivity of the preparation available.

The paper chromatography technique was applied in the two-dimensional ascending technique in both qualitative and quantitative analyses, as recommended in Pabst Circular OR-10 (84). The application of the method for the present investigation was explained earlier.

The spectrophotometrically determined increase in extinction at 260 mμ after uranyl acetate perchloric acid precipitation was used to register the formation of acid soluble degradation products, and the course and suitable speed of the hydrolysis was ensured by other methods. The extinction measurements of the filtrates were made from 0.15 : 5.0 dilutions in a quartz cell 1 cm in diameter.

Enzymes. — Armillariella mellea and Lactarius torminosus lyophilisates were the enzymes used for analysis of the degradation products. The preparations were dissolved in distilled water giving a stock solution concentration mostly of 2 per cent used in the tests either as such or diluted. The pH of the enzyme solutions was 5.0.

Substrates. — The substrate most commonly used was Light & Co's ribonucleic acid which was purified by precipitating twice with acetic acid. The preparation obtained was faultless. Its properties and analysis were described earlier. In addition to the above substance, use was made in some tests of ribonucleic acid labeled with P^{32} isotope. The preparation was made on special request by Abbot Laboratories, North Chicago, Ill. For routine phosphorus analyses a 0.125 per cent stock solution of Light & Co's ribonucleic acid was made in Michaelis buffer, final pH 3.85. For the determination of radioactive phosphorus a 2 per cent solution was made of the isotope preparation in Michaelis buffer, final pH likewise 3.85. This radioactive stock solution was used as such both for experiments with the liberation of inorganic radioactive phosphorus and for some chromatographic analyses. The solution thus concentrated was also needed for the phosphorus analyses because the radioactivity of the preparation was relatively low, 0.18 mc. per gm. For routine chromatographic analyses a 2 per cent solution, pH 3.85, was prepared in the same way from a purified Light & Co preparation.

EXPERIMENTAL PROCEDURE AND RESULTS

LIBERATION OF INORGANIC PHOSPHORUS

Ordinary ribonucleic acid (Light & Co., Ltd., twice purified) as the substrate. — The liberation of inorganic phosphorus from this substrate was studied with an Armillariella mellea preparation and a Lactarius torminosus preparation. The following solutions were pre-heated in a water bath at +37 °C for 10 min. in identical conditions and pipetted into the test tubes:

Reagent:		*Test:*	*Blank:*	*Control:*
Substrate:	RNA, 1.25 mg/ml, pH 3.85	12 ml	—	12 ml
Enzyme:	Armillariella mellea resp. Lactarius torminosus, 5 mg/ml, pH 5.0	3 „	3 ml	—
Buffer:	Michaelis buffer, pH 3.85	—	12 „	3 „

The final concentrations of the reaction mixture were 0.1 per cent both in the substrate and the enzyme, and the final pH was 4.0. Before it was possible to follow the inorganic

TABLE 4

THE PHOSPHORUS CONTENT OF THE COMPONENTS OF THE REACTION MIXTURE BEFORE THE BEGINNING OF ENZYME DIGESTION USING A 0.1 PER CENT SUBSTRATE CONCENTRATION AND A 0.1 PER CENT ENZYME CONCENTRATION.

Reaction Mixture Component	Reaction Mixture (*Lactarius torminosus*)			Reaction Mixture (*Armillariella mellea*)		
	Total Phosphorus µg/ml	Organic Phosphorus µg/ml	Inorganic Phosphorus µg/ml	Total Phosphorus µg/ml	Organic Phosphorus µg/ml	Inorganic Phosphorus µg/ml
RNA (Light & Co., Ltd., twice purified)	73.1	73.1	—	73.1	73.1	—
Fungus enzyme (lyophilised)	7.2	—	7.2	8.1	—	8.1
	80.3	73.1	7.2	81.2	73.1	8.1

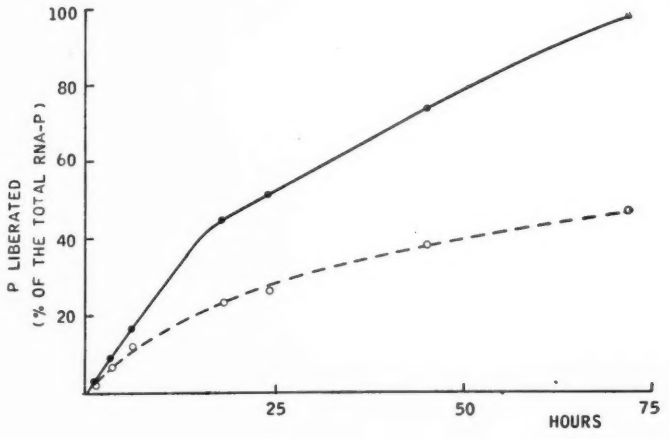

Fig. 16. — Conversion of the phosphorus of ribonucleic acid into inorganic form under the influence of two fungus enzymes. Reaction mixture and reaction conditions identical with those of the experiments reported in Fig. 17.

——•——•—— Inorganic phosphorus liberated under the influence of Armillariella mellea fungus enzyme.

– – –o– – –o Inorganic phosphorus liberated under the influence of Lactarius torminosus fungus enzyme.

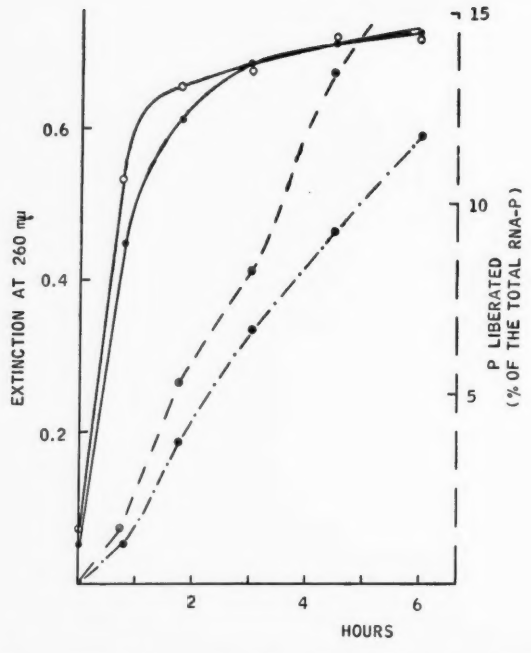

Fig. 17. — Liberation of inorganic phosphorus under the influence of two fungus enzymes. Reaction mixture: RNA (Light & Co., Ltd., twice purified) 1.25 mg/ml, 12.0 ml. Enzyme 5 mg/ml, 3.0 ml. Reaction mixture pH 4.0 in Michaelis buffer. Reaction time 6 hours. Reaction temperature + 37 °C. The figure also shows the changing of the substrate to acid-soluble form, determined in identical conditions and from identical samples.

- Inorganic phosphorus liberated under the influence of Armillariella mellea fungus enzyme.
- Inorganic phosphorus liberated under the influence of Lactarius torminosus fungus enzyme.
- Acid-soluble degradation products originating under the influence of Armillariella mellea fungus enzyme.
- Acid-soluble degradation products originating under the influence of Lactarius torminosus fungus enzyme.

phosphorus liberated in the reaction the phosphorus contents of the components of the reaction mixture had to be analysed. The results are given in Table 4.

With the phosphorus contents of the reaction mixture components known, the inorganic phosphorus liberated under the influence of the enzyme hydrolysis was determined from samples taken at suitable intervals from the 0 moment. 3 ml samples

were precipitated with 0.6 ml uranyl acetate perchloric acid reagent and then filtered. Extinction was measured at 260 mμ from a 0.15 : 5.0 dilution of the filtrates against the corresponding blank solution. It was thus possible to follow the course of the reaction by an already familiar and reliable technique. The remainder of the filtrate was employed in the determination of inorganic phosphorus. The dye developed was measured spectrophotometrically in quartz cells 0.5 cm in diameter at 730 mμ. It was possible in this way to calculate the amount of inorganic phosphorus per sample and, from it, per ml of the reaction mixture. By deducting the amount in the reaction mixture components from this value the amount of inorganic phosphorus liberated by enzyme hydrolysis could be expressed in per cent of the total phosphorus of the substrate. Parallel tests were made throughout of a reaction mixture similar except that the enzyme was omitted.

Figs. 16 and 17 show the liberation of inorganic phosphorus during digestion of ribonucleic acid with fungus enzymes. It will be seen from Fig. 16 how the Armillariella mellea preparation was able within 75 hours to liberate nearly all the substrate phosphorus in inorganic form, while the Lactarius torminosus preparation capable in identical conditions of liberating only about a half in the same time. In spite of this difference, both fungus enzymes changed the substrate into an acid-soluble form in almost exactly the same time. Fig. 17 shows the parallel determination of the increase in extinction at 260 mμ caused by acid-soluble degradation products. It reveals how the liberation of inorganic phosphorus occurs later than the formation of acid soluble degradation products. When the substrate in its entirety was acid-soluble, indicated when the increase in extinction ceased at 260 mμ, the amount simultaneously liberated from the total phosphorus of the substrate was only slightly over 10 per cent whichever fungus enzyme was used. However, there was a distinct difference between the fungus enzymes in the measurements of liberated inorganic phosphorus when the enzyme hydrolysis was observed for a longer time.

Isotope ribonucleic acid (RNA-P^{32}, Abbot Laboratories) as the substrate. — The liberation of inorganic phosphorus from this

substrate was examined with a Lactarius torminosus preparation. The following solutions pre-heated for 10 min. in a water bath at +37 °C in the same conditions were pipetted into the test tubes:

Reagent:		*Test:*	*Control:*
Substrate:	RNA-P^{32}, 20 mg/ml, pH 3.85	10 ml	10 ml
Enzyme:	Lactarius torminosus, 20 mg/ml, pH 5.0	10 „	—
Buffer:	Michaelis buffer, pH 3.85	—	10 „

The final concentrations of the reaction mixture components, both substrate and enzyme, were thus 1.0 per cent, and the final pH was 4.0. Before starting the reaction, the substrate was checked for inorganic phosphorus. None was found. The inorganic radioactive phosphorus liberated by enzyme hydrolysis was then determined from samples taken at suitable time intervals from the 0 moment. The radioactivity was measured as follows: 1.0 ml of the reaction mixture was precipitated with the same quantity of acid alcohol. The precipitate formed was filtered immediately through Whatman No.50 filter paper. The filtrate was evaporated in sample pans with an infra red lamp at +80 — +90 °C, avoiding distillation. The radioactivity of the evaporated filtrate was measured in the sample pans to accurate geometric standards. In the initial stage of the hydrolysis the time expended on the registration of 2000 impulses and in a later phase the time expended on the registration of 10^4 impulses were observed. In the former case the probable measuring error was 2.25 per cent and in the latter 1.0 per cent.

The radioactivity of the sediment on filter paper was determined from each sample. It was not possible to do the measuring according to equally strict geometric standards, and this is reflected in the results. In the precipitate measurements the time expended on the registration of 10^4 impulses was observed throughout but the measuring error was difficult to assess.

Fig. 18 shows the liberation of radioactive inorganic phosphorus from isotope ribonucleic acid by fungus enzyme hydrolysis. Attention is drawn to the slightly more rapid liberation of inorganic radioactive phosphorus from the isotope substrate than from the ordinary ribonucleic acid. Within 5 hours of the start of the reaction it showed signs of ceasing and within 24 hours no further inorganic radioactive phosphorus was liberated.

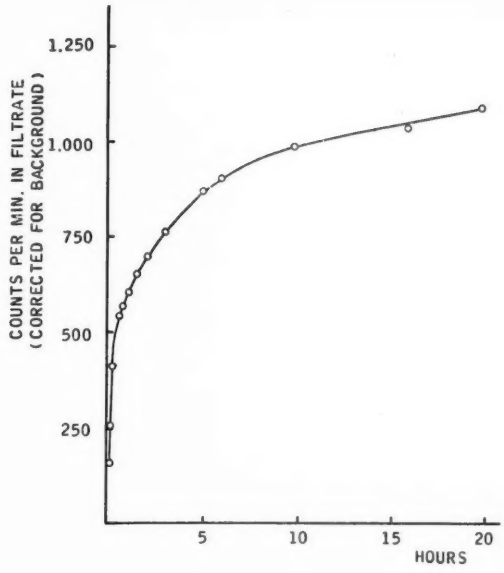

Fig. 18. — Liberation of radioactive inorganic phosphorus from isotope ribonucleic acid (RNA—P^{32}) under the influence of Lactarius torminosus fungus enzyme. Reaction mixture: RNA—P^{32}, 20 mg/ml, 10.0 ml. Enzyme, 20 mg/ml, 10.0 ml. Reaction mixture pH 4.0 in Michaelis buffer. Reaction time 24 hours. Reaction temperature +37 °C.

Control determinations of the liberation of inorganic phosphorus with active and inactive fungus enzyme. — Although all the preceding control tests showed that there was no spontaneous liberation of inorganic phosphorus from ribonucleic acid unless an enzyme was present, it seemed to be of interest to ascertain whether inorganic phosphorus liberation was associated solely with enzyme activity or whether there were other factors of potential error, especially when the active actual enzyme was destroyed but the enzyme protein remained in undenatured form in the reaction mixture.

The heat stability studies showed that, assessed on the basis of the turbidity reaction, 0.45 per cent Armillariella mellea solution heated for 2 hours at +70 °C had an activity equal to 0.05 per cent of the activity of unheated Armillariella mellea solution. With this in mind, the enzyme concentrations of the reaction mixture were varied after the initial reaction of one

TABLE 5

EXPERIMENTAL PROCEDURE FOR STUDYING THE DEPENDENCE OF THE LIBERATION OF INORGANIC PHOSPHORUS ON THE FLUCTUATIONS OF THE CONCENTRATIONS OF THE FUNGUS ENZYME (ARMILLARIELLA MELLEA) IN THE REACTION MIXTURE DURING INCUBATION.

Noting of the Reaction	Added to the Reaction Mixture 1 hr after Incubation (Equal Volumes)	Substrate Concentration of the Reaction Mixture, %	Enzyme Concentration of the Reaction Mixture		
			Active Enzyme %	Inactive Enzyme %	Total Enzyme %
Initial reaction	—	0.1	0.1	—	0.1
Further reaction I	distilled water	0.05	0.05	—	0.05
Further reaction II	active enzyme, 0.1 %	0.05	0.1	—	0.1
Further reaction III	inactive enzyme, 0.1 %	0.05	0.05	0.05	0.1
Further reaction IV	active enzyme, 0.9 %	0.05	0.5	—	0.5
Further reaction V	inactive enzyme, 0.9 %	0.05	0.05	0.45	0.5

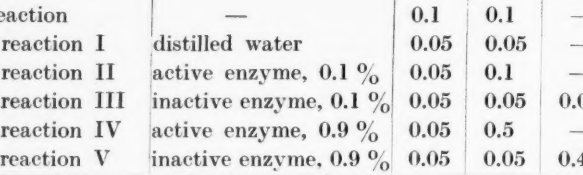

Fig. 19. — The dependence of the liberation of inorganic phosphorus on the variation of the Armillariella mellea fungus enzyme concentrations in the reaction mixture. The experimental procedure is detailed in Table 5. Reaction mixture pH 4.2 Reaction time 24 hours. Reaction temperature +37 °C.

Initial reaction
Further reaction I
Further reaction II
Further reaction III
Further reaction IV
Further reaction V

hour in which the substrate and enzyme concentrations were both 0.1 per cent. By making these changes in the enzyme concentrations it was possible to establish simultaneous variations in the liberation of inorganic phosphorus.

Table 5 gives the experimental procedure for adding enzyme to the reaction mixture after one hour's initial reaction and thus causing fluctuations in the enzyme concentration in the middle of the reaction. For the sake of clarity, only the names given in the table for the changed reactions will be used below. Initial reaction refers here to a reaction mixture in which the enzyme and substrate concentrations are the same (0.1 per cent). The pH of all the reaction mixtures was kept at pH 4.0 as accurately as possible. External conditions were carefully standardised so as to be identical for all the reactions. Armillariella mellea lyophilisate was the enzyme preparation used.

Fig. 19 gives the results of the fluctuations in the liberation of inorganic phosphorus caused by the different enzyme concentrations of the reaction mixture. It appeared that when the initial reaction was unchanged and the enzyme and substrate concentrations were 0.1 per cent, 51 per cent of the total phosphorus of the ribonucleic acid in the reaction mixture was liberated within 24 hours as inorganic phosphorus.

If the initial reaction was changed one hour after the beginning of incubation by adding the same amount of distilled water to the reaction mixture both the enzyme and substrate concentrations fell to half (0.05 per cent) of the original (0.1 per cent). This notwithstanding, nearly the same amount, in this case 43 per cent, of the total phosphorus of the ribonucleic acid of the reaction mixture was liberated in 24 hours as inorganic phosphorus (further reaction I).

If the initial reaction was changed one hour after the beginning of incubation by adding to the reaction mixture the same amount of 0.1 per cent enzyme solution heated for 2 hours at +70 °C, the concentrations of the substrate and active enzyme fell to half (0.05 per cent) of the original (0.1 per cent) although the total enzyme concentration remained unchanged (0.1 per cent). In this case (further reaction III) 50 per cent, i.e. again an amount corresponding to that in the initial reaction and further reaction I, of the total phosphorus of the ribonucleic acid in the reaction

mixture was liberated in 24 hours as inorganic phosphorus.

If the initial reaction was changed one hour after the beginning of incubation by adding to the reaction mixture the same amount of 0.9 per cent enzyme solution heated for 2 hours at +70 °C (according to the earlier experiments, with activity diminished to roughly 0.1 per cent of active enzyme), the concentrations of the substrate and active enzyme fell to half (0.05 per cent) of the original (0.1 per cent) although the total enzyme quantity remained high (0.5 per cent). In this case (further reaction V), too, a similar result was achieved to that in further reaction I and III: 54 per cent of the total phosphorus of the ribonucleic acid in the reaction mixture was liberated in 24 hours as inorganic phosphorus.

In these four varied tests, in all of which the concentrations of active enzyme and substrate in the reaction mixture were calculated or assessed to be the same, the amount of inorganic phosphorus liberated was largely similar (51, 43, 50 and 54 per cent). This notwithstanding the fact that the reaction mixture contained additional enzyme protein inactivated by heat treatment but nevertheless undenatured.

If the initial reaction was changed one hour after the beginning of incubation by adding to the reaction mixture the same amount of 0.1 per cent active enzyme, the substrate concentration fell to half (0.05 per cent) of the original (0.1 per cent) but the proportion of active enzyme remained unchanged (0.1 per cent), 70 per cent of the total phosphorus of the ribonucleic acid in the reaction mixture was liberated in 24 hours as inorganic phosphorus (further reaction II).

If the initial reaction was changed one hour after the beginning of incubation by adding to the reaction mixture the same amount of 0.9 per cent active enzyme, the substrate concentration fell to half (0.05 per cent) of the original (0.1 per cent) but the amount of active enzyme increased five times (0.5 per cent). Within 9 hours 90 per cent of the total phosphorus of the ribonucleic acid in the reaction mixture was liberated as inorganic phosphorus (further reaction IV).

In these two further reactions (II and IV), in which the concentration of active enzyme was raised but the substrate concentrations kept the same (although a half smaller than in the

initial reaction), the amount of inorganic phosphorus liberated from the ribonucleic acid in the reaction mixture rose sharply.

To summarise the tests with active and inactive enzyme, liberation of inorganic phosphorus from the ribonucleic acid in the reaction mixture depends solely and very sensitively on the concentration of the enzyme in the reaction mixture, but only when the enzyme is active. On the other hand, even a heavy addition of only just inactivated but undenatured enzyme protein causes no cleavage of inorganic phosphorus from the substrate except under the influence of a part that has remained accidentally undestroyed.

QUALITATIVE CHROMATOGRAPHIC STUDIES OF THE LIBERATED NUCLEIC ACID DERIVATIVES

Reaction mixture. — The results of ribonucleic acid hydrolysis obtained by various methods in the foregoing were checked chromatographically against the following standard reaction mixture. Lactarius torminosus lyophilisate alone was the enzyme used because it does not interfere with ultraviolet light on paper in contrast to Armillariella mellea preparation. The following solutions pre-heated for 10 min. in a water bath at +37 °C in identical conditions were pipetted into the test tubes:

Reagent:		*Test:*	*Control:*
Substrate:	RNA, 20 mg/ml, pH 3.85	1.5 ml	1.5 ml
Enzyme:	Lactarius torminosus, 20 mg/ml, pH 5.0	1.5 „	—
Buffer:	Michaelis buffer, pH 3.85	—	1.5 „

The concentration of both substrate and enzyme in the reaction mixture was thus 1.0 per cent, and the pH of the reaction mixture 4.0. Depending on the experiment, suitable amounts of the sample were pipetted onto paper after a sufficiently long hydrolysis. The time at which the samples were taken was generally determined by the preliminary tests of which a good many had to be made. A nonhydrolysed control sample was obtained from the control tube with no enzyme in order to discover whether there was any spontaneous hydrolysis at the

beginning and end of the reaction. If the quantity of the sample was small and easily absorbable in paper, no endeavour was made to stop the reaction. If, on the other hand, a large quantity of sample was used and it was absorbed slowly in paper the reaction mixture was moved at the moment of taking the sample from the water bath into ice alcohol mixture. The reaction mixture was kept in the latter conditions at the margin of freezing in order to stop enzyme activity at the desired moment.

Depolymerisation degree. — To determine the starting moment of fungus ribonucleolytic action the samples taken at different time intervals were analysed by the single dimensional ascending paper chromatography technique using two solvents.

With 0.1 M phosphate buffer pH 6.8 — ammonium sulphate — n-propanol solvent the sample quantity was generally 20 μl and the running time 10 hours at +27 °C. This solvent was used to obtain a general idea of enzyme hydrolysis.

With n-butanol — water solvent the sample quantity was 10 μl and the running time 35 hours at +20 °C. This solvent moves only degradation products smaller than the nucleotide degree. The first samples were taken at 10 min. intervals in both the systems. The time for taking the future samples was determined by preliminary tests. Spontaneous hydrolysis was controlled at the beginning and end of the test against the enzyme-free control tube.

The results of the tests are shown in Figs. 20 and 21. They reveal the incipient degradation of ribonucleic acid as a function of the time of digestion by Lactarius torminosus lyophilisate. Chromatographically demonstrable degradation products smaller than the nucleotide degree were established in these conditions in the reaction mixture very soon after the beginning of digestion.

Mononucleotide degree. — An endeavour was made to register the nucleotide degree of ribonucleic acid hydrolysis produced by fungus enzyme. It consisted of taking from the reaction mixture samples of 50 μl at 5-minute intervals from the start up to one hour and, then, at hourly intervals up to the total reaction time of 24 hours. The samples were analysed two-dimensionally using n-propanol — ammonia — water as the solvent in the first dimension and a running time with the descending technique

of 55 hours at +20 °C. The solvent of the second dimension was ammonium sulphate — isopropanol — water, and the running time with the ascending technique 10 hours at +20 °C.

The behaviour of the known 5'-ribonucleotides (Pabst Laboratories) was determined in the same conditions and with the same solvents. The nucleotides were dissolved in Sörensen's neutral phosphate buffer to form 0.5 per cent stock solutions from which 5 µl was pipetted onto the starting point. Fig. 22 shows the charting of these control nucleotides.

When the fungus enzyme hydrolysis of the ribonucleic acid was analysed as outlined above, no chromatographically demonstrable nucleotides (Figs. 23 and 24) were established in the hydrolysate at any stage of the reaction or among the final products of the reaction. However, the result was the same when the solvent systems of the second dimension were varied over the range given in Pabst Circular OR-10 (84). The nucleotides obviously originated in all probability as intermediary products of the reaction and were rapidly hydrolysed further into smaller degradation products.

Nucleoside degree. — The chromatographic method of isolating digestion products used was n-butanol — water solvent because it is capable of isolating selectively degradation products smaller than the nucleotide degree. It was natural to combine this solvent with 0.1 M phosphate buffer pH 6.8 — ammonium sulphate — n-propanol and with ethanol — 1 M ammonium acetate pH 7.5 and with isobutyric acid — conc. ammonium hydroxide — water in two-dimensional paper chromatography analysis. Continuous enzyme hydrolysis for 24 hours seemed the most suitable method of obtaining even, uniform and reliable results. 25 µl of hydrolysate proved to be a suitable amount both because it permitted quick pipetted and since the degradation products were readily mobile and no tailing occurred. In that concentration they had a similar ultraviolet absorption on chromatograms to the known 5 µl control spots of the 0.5 per cent nucleoside or nucleotide stock solution. The uniform pipetting scheme was employed in all the principal analyses. This facilitated assessment of the results. All the runs were performed with at least three solvent systems in the second dimension, chosen to give known nucleoside and nucleotide

rf-values that differed completely from one another. The runs were very carefully made in an accurately calibrated thermostat at +27 °C. Thanks to these precautions the spots were clear and no trace spots originated. Known purine and pyrimidine bases (Krishell Laboratories), ribonucleosides and ribonucleotides (Pabst Laboratories) were used as controls. The chromatograms were developed by the ultraviolet printing technique on photographic paper.

Table 6 and Figs. 25, 26, 27, 28, 29 and 30 show the results of this isolation method parallel with those of the known nucleoside and nucleotide controls. It would seem by a rough estimate that the majority of the end products of the fungus enzyme hydrolysis of ribonucleic acid consists of degradation products comparable primarily with nucleosides. It is possible by the same isolation method to isolate these degradation products from their hydrolysate for closer examination and identification.

Identification of the digestion products by means of their rf-values. — The corresponding rf-values for the controls (spots 2, 3, 4, 5 and 6) were calculated from the two-dimensional chromatography analyses (spots 7a, 7b, 7c and 7d) of the fungus enzyme hydrolysates of ribonucleic acid presented in Figs. 25, 26 and 27. Table 6 contains the results and comments on the chromatographic technique. The table also shows the rf-values in the literature of authentic ribonucleosides and ribonucleotides (Pabst Laboratories) when these solvent systems are used. It further contains the rf-values obtained from the corresponding authentic ribonucleosides and ribonucleotides by the technique employed here. Finally, the table gives the corresponding rf-values of the degradation products isolated from the fungus enzyme hydrolysis of ribonucleic acid. The rf-values of the degradation products were in good agreement with the corresponding figures for authentic ribonucleosides. This confirmed the opinion that these degradation products have a ribonucleoside character.

Identification of the digestion products by means of their specific ultraviolet spectra. — The degradation products isolated from fungus enzyme hydrolysate (Figs. 25, 26 and 27) were further studied by determining their ultraviolet spectra from a chromatographic paper corresponding to one of the solvent systems employed. Each spot was localised, cut off, cut up and eluated for 2 hours by shak-

CHROMATOGRAPHIC ANALYSIS OF HYDROLYSIS END PRODUCTS LIBERATED IN 24 HOURS FROM RIBONUCLEIC ACID UNDER THE INFLUENCE OF LACTARIUS TORMINOSUS FUNGUS ENZYME COMPARED WITH AUTHENTIC CONTROLS. (REACTION MIXTURE: SUBSTRATE CONCENTRATION 1.0 PER CENT, FUNGUS ENZYME CONCENTRATION 1.0 PER CENT, PH 4.0, TEMPERATURE +37 °C)

Number of Solvent Systems	Technical Data on Two-dimensional Chromatography Assay		Authentic Samples								Samples isolated from Hydrolysate	
			5'-ribonucleotides	The rf-values of Nucleotides*)			Ribo-nucleosides	The rf-values of Nucleosides*)		Noting of the Spot (in Figs. 25, 26, 27)	The rf-values Obtained by the Author
	First Dimension	Second Dimension		In the Literature		Obtained by the Author		In the Literature	Obtained by the Author		
I (Figs. 25, 28)	n-butanol-water, 86/14, ascending technique, 36 hrs, +20 °C. *Spot:* 25 μl of reaction mixture	0.1 M phosphate buffer, pH 6.8 — ammonium sulphate — n-propanol, 100/60/2, ascending technique, 14 hrs, +27 °C.	AMP, ADP, ATP	0.29 0.37 0.48		0.29 0.35 0.42	Adenosine	0.13	0.13	7 a	0.12
			GMP, GDP, GTP	0.50 0.58 0.61		0.49 0.57 0.63	Guanosine	0.34	0.30	7 b	0.31
			IMP, IDP, ITP	0.57 0.63 0.70		0.53 0.60 0 64	Inosine	0 41	0.39	—	—
			UMP, UDP, UTP	0.64 0.71 0.77		0.67 0.72 0.76	Uridine	0.54	0.56	7 c	0.58
			CMP, CDP, CTP	0.67 0.71 0.77		0.66 0.73 0.78	Cytidine	0.56	0.58	7 d	0.58
II (Figs. 26, 29)	—,,—	Ethanol—1.0 M ammonium acetate pH 7.5, 7/3, ascending technique, 16 hrs, +27 °C.	AMP, ADP, ATP	0.15 0.07 0.04		0.15 0.07 0.04	Adenosine	0.63	0.63	7 a	0.64
			GMP, GDP, GTP	0.09 0.06 0.03		0.09 0.05 0.03	Guanosine	0.56	0.56	7 b	0.59
			IMP, IDP, ITP	— — —		0.12 0.06 0.04	Inosine	—	0.63	—	—
			UMP, UDP, UTP	0.18 0.09 0.04		0.20 0.11 0.06	Uridine	0.73	0.73	7 c	0.73
			CMP, CDP, CTP	0.13 0.07 0.04		0.15 0.08 0.05	Cytidine	0.67	0.67	7 d	0.68
III (Figs. 27, 30)	—,,—	Isobutyric acid—conc. ammonium hydroxide—water, 66/1/33, pH 3.7, ascending technique, 24 hrs, +27 °C. *Spot:* authentic control, 0.5 per cent, pH 7.0, 5 μl; reaction mixture, 15 μl.	AMP, ADP, ATP	0.45 0.30 0.20		0.43 0.29 0.21	Adenosine	0.76	0.78	7 a	0.78
			GMP, GDP, GTP	0.13 0.09 0.05		0.15 0.09 0.06	Guanosine	0.43	0.41	7 b	0.41
			IMP, IDP, ITP	0 19 0.12 0.10		0.19 0.10 0.08	Inosine	0.47	0.42	—	—
			UMP, UDP, UTP	0.23 0.13 0.08		0.23 0.11 0.07	Uridine	0.47	0.43	7 c	0.41
			CMP, CDP, CTP	0.28 0.13 0.07		0.33 0.15 0.11	Cytidine	0.59	0.59	7 d	0.59

*) in second dimension

ing mechanically at +37 °C in 2 ml of 0.1 N HCl. The ultraviolet spectra of the neutralised eluates outside the range 220—320 mμ were measured from suitable dilutions. The extinctions were read against the solution used in eluation. At the same time, from the immediate vicinity of each spot and from the same rf-value range, an equally large area of paper was eluated for blank values to assist in correcting the analysis extraction values.

Fig. 31 shows the ultraviolet spectra of authentic ribonucleosides in phosphate buffer pH 7.0 and in a suitable concentration for purpose of comparision. Fig. 32 shows the ultraviolet spectra of the degradation products isolated from the

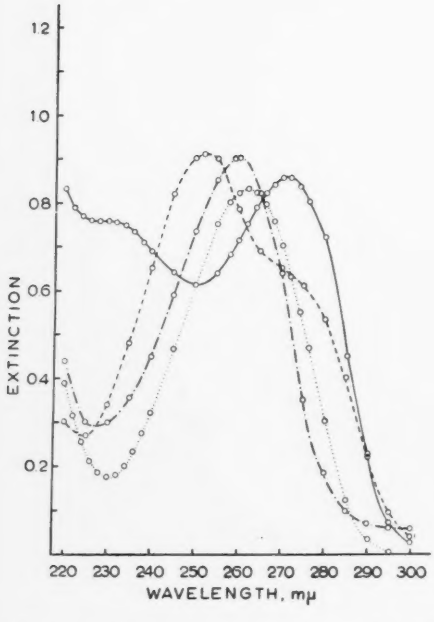

Fig. 31. — The ultraviolet spectra of authentic ribonucleosides (Pabst Laboratories) in phosphate buffer pH 7.0

Adenosine
Guanosine
Uridine
Cytidine

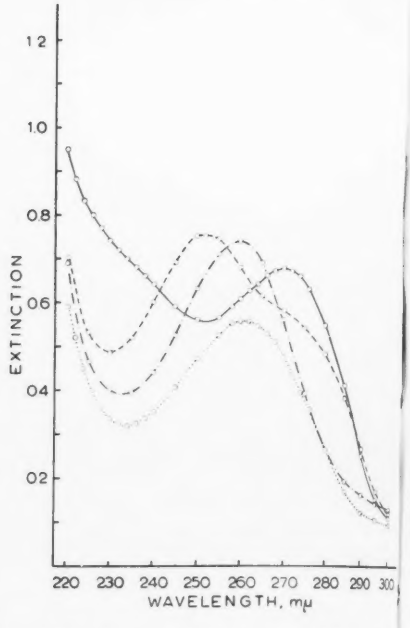

Fig. 32. — The degradation products liberated i hours from ribonucleic acid under the influenc the Lactarius torminosus fungus enzyme. Elution performed from the chromatogram presented Fig. 25.

Ultraviolet spectrum of spot 7
" " " " 7
" " " " 7
" " " " 7

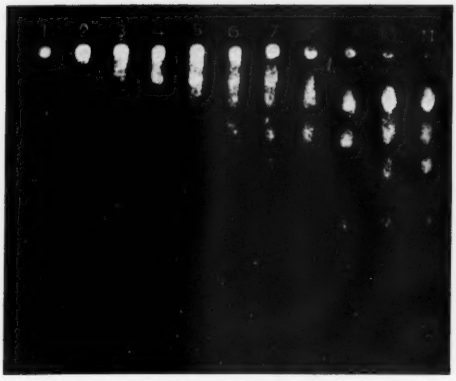

Fig. 20. — The gradual degradation of ribonucleic acid with the increase in digestion time under the influence of Lactarius torminosus fungus enzyme. Reaction mixture: RNA (Light & Co., Ltd., twice purified), 20 mg/ml, 2.0 ml. Enzyme, 20 mg/ml, 2.0 ml. Reaction mixture, pH 4.2, in Michaelis buffer. Reaction temperature +37 °C.
Solvent: 0.1 M phosphate buffer pH 6.8 — ammonium sulphate — n-propanol, 100/60/2, 10 hours, at +27 °C with the ascending technique. Spot: 20 μl of hydrolysate.

No. of spot	1	2	3	4	5	6	7	8	9	10	11
Digestion time:	Undigested RNA-control	0	10	20	30	60	2 $^1/_2$	5	10	15	20
		minutes						hours			

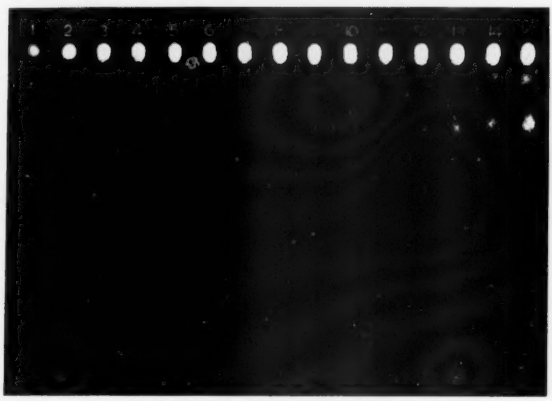

Fig. 21. — The gradual degradation of ribonucleic acid with the increase in digestion time under the influence of Lactarius torminosus fungus enzyme. The reaction mixture was given in Fig. 20.
Solvent: n-butanol — water, 86/14, 35 hours, at +20 °C with the ascending technique. Spot: 20 μl of hydrolysate

No. of spot	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Digestion time, minutes:	Undigested RNA-control	0	10	20	30	40	50	60	70	80	90	100	110	120	180

Fig. 22

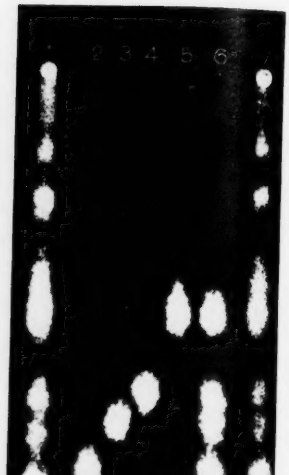

Fig. 23

Fig. 24

Fig. 22. — Two-dimensional chromatographic system for the separation of authentic 5'-ribonucleotides.

Solvents: 1-dimension (A): n-propanol—ammonia—water, 60/30/10, 55 hours, at +20 °C with the descending technique. 2-dimension (B): ammonium sulphate — isopropanol — water, 79/2/19, 10 hours, at +27 °C with the ascending technique. Spots: 5 μl of each nucleotide (0.5 per cent, pH 7.0).

No. of spot	1	2	3	4	5	6	7	8	9	10	11	12
Nucleotide:	AMP	ADP	ATP	GMP	GDP	GTP	IMP	IDP	ITP	CMP + UMP	CDP + UDP	CTP + UTP

Fig. 23. — One-dimensional chromatogram of the degradation of ribonucleic acid by Lactarius torminosus fungus enzyme in 24 hours. Included in the figure are one dimensional control runs of authentic ribonucleosides. The reaction mixture was given in Fig. 20.

Solvent: n-propanol—ammonia—water, 60/30/10, 55 hours, at +20 °C with the descending technique. Spots: authentic ribonucleoside controls, 5 μl (0.5 per cent, pH 7.0); hydrolysate, 15 μl.

No. of spot	1	2	3	4	5	6	7
Substance:	Hydrolysate	Adenosine	Cytidine	Uridine	Guanosine	Adenosine + Cytidine + Uridine + Guanosine	Hydrolysate

Fig. 24. — Two-dimensional chromatogram of the degradation of ribonucleic acid by Lactarius torminosus fungus enzyme in 24 hours. The reaction mixture was given in Fig. 20.

Solvents: 1-dimension: (A): n-propanol—ammonia—water, 60/30/10, 55 hours, at +20 °C with the descending technique. 2-dimension (B): ammonium sulphate—isopropanol—water, 79/2/19, 10 hours, at +27 °C with the ascending technique. Spot: 50 μl of hydrolysate.

On a simultaneously run control chromatogram the authentic ribonucleosides pipetted onto the starting point (5 μl of each nucleoside; 0.5 per cent, pH 7.0) moved as follows in relation to the spots of the analysis chromatogram:

No. of spot (hydrolysate)	1	2	3	4
Authentic ribonucleoside:	Guanosine	Adenosine	Uridine	Cytidine

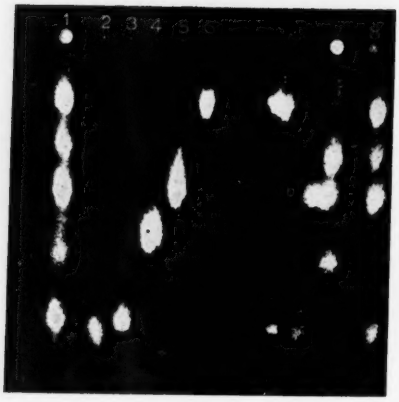

Fig. 25

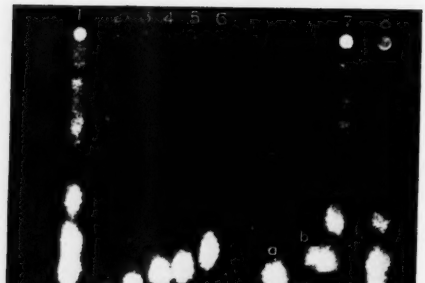

Fig. 26

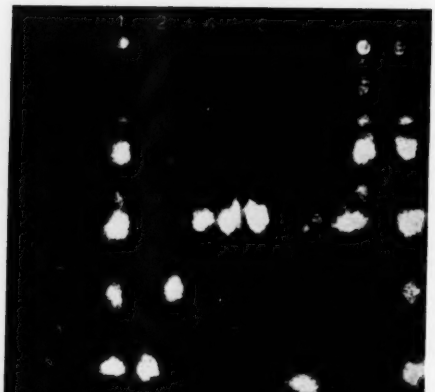

Fig. 27

Fig. 25. — Two dimensional chromatogram of the degradation of ribonucleic acid by Lactarius torminosus fungus enzyme in 24 hours. Included in the figure are one-dimensional runs of the same hydrolysate and authentic ribonucleosides. The reaction mixture was given in Fig. 20. The rf-values are given in Table 6.

Solvents: 1-dimension: n-butanol—water, 86/14, 36 hours, at +20 °C, with the ascending technique. 2-dimension: 0.1 M phosphate buffer pH 6.8—ammonium sulphate—n-propanol, 100/60/2, 14 hours, +27 °C with the ascending technique. Spots: authentic ribonucleoside controls, 5 μl (0.5 per cent, pH 7.0). One-dimensional runs of the hydrolysate controls, 15 μl. Principal analysis of two-dimensional run of the hydrolysate, 25 μl.

No. of spot	1	2	3	4	5	6	7 a	7 b	7 c	7 d	8
Substance:	Hydrolysate control, 1-dim.technique	Cytidine ,,	Uridine ,,	Inosine ,,	Guanosine ,,	Adenosine ,,	Principal hydrolysate analysis, 2-dim.technique				Hydrolysate control, 1-dim.technique

Fig. 26. — Two-dimensional chromatogram of the degradation of ribonucleic acid by Lactarius torminosus fungus enzyme in 24 hours. Included in the figure are one-dimensional control runs of the same hydrolysate and authentic ribonucleosides. The experimental procedure applied in the analysis is similar to that in Fig. 25. The rf-values are given in Table 6.

Solvents: 1-dimension: n-butanol—water, 86/14, 36 hours, at + 20 °C, with the ascending technique. 2-dimension: ethanol—1.0 M ammonium acetate pH 7.5, 7/3, 16 hours, at +27 °C with the ascending technique. Spots: authentic ribonucleoside controls, 5 μl (0.5 per cent, pH 7.0). One dimensional runs of the hydrolysate controls, 15 μl. Principal analysis of two-dimensional run of the hydrolysate, 25 μl

No. of spot	1	2	3	4	5	6	7 a	7 b	7 c	7 d	8
Substance:	Hydrolysate control, 1-dim.technique	Uridine ,,	Cytidine ,,	Adenosine ,,	Inosine ,,	Guanosine ,,	Principal hydrolysate analysis, 2-dim.technique				Hydrolysate control, 1-dim.technique

Fig. 27. — Two-dimensional chromatogram of the degradation of ribonucleic acid by Lactarius torminosus fungus enzyme in 24 hours. Included in the figure are one-dimensional control runs of the same hydrolysate and authentic ribonucleosides. The experimental procedure applied in the analysis is similar to that in Fig. 25. The rf-values are given in Table 6.

Solvents: 1-dimension: n-butanol—water, 86/14, 36 hours, at +20 °C with the ascending technique. 2-dimension: isobutyric acid—conc. ammonium hydroxide — water, 57/4/39, 24 hours, at +27 °C with the ascending technique. Spots: authentic ribonucleoside controls, 5 μl (0.5 per cent, pH 7.0). One-dimensional runs of the hydrolysate controls, 15 μl. Principal analysis of two-dimensional run of the hydrolysate, 25 μl.

No. of spot	1	2	3	4	5	6	7 a	7 b	7 c	7 d	8
Substance:	Hydrolysate control, 1-dim.technique	Adenosine ,,	Cytidine ,,	Uridine ,,	Guanosine ,,	Inosine ,,	Principal hydrolysate analysis, 2-dim.technique				Hydrolysate control, 1-dim.technique

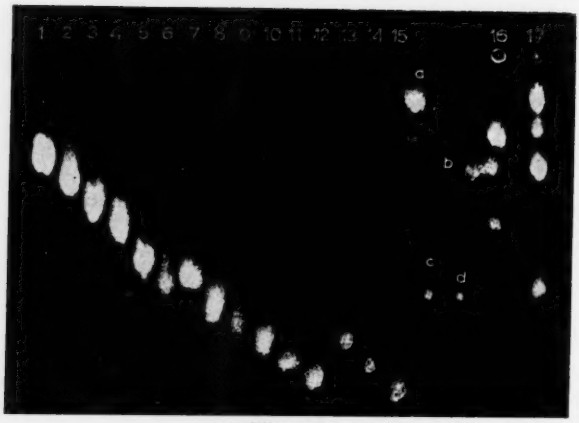

Fig. 28

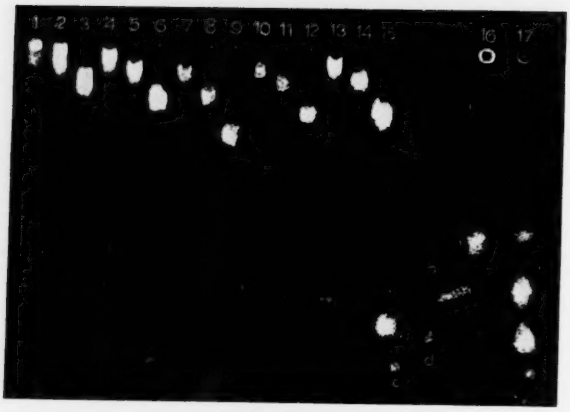

Fig. 29

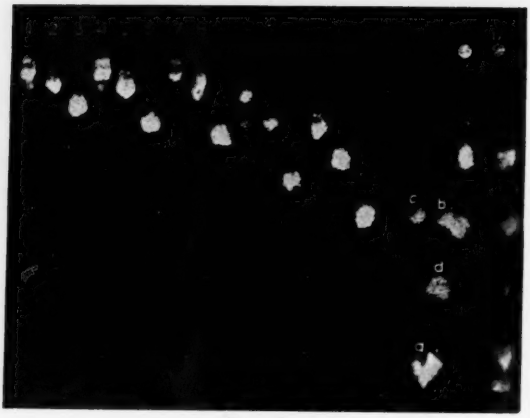

Fig. 30

Fig. 28. — Two-dimensional chromatogram of the degradation of ribonucleic acid by Lactarius torminosus fungus enzyme in 24 hours. Included in the figure are one-dimensional control runs of the same hydrolysate and authentic 5'-ribonucleotides. The experimental procedure, solvents and concentrations of the spots applied in the analysis are similar to that in Fig. 25. The rf-values are given in Table 6.

No. of spot	1	2	3	4	5	6	7	8	9	10
Substance:	AMP	ADP	ATP	GMP	GDP	GTP	IMP	IDP	ITP	UMP

11	12	13	14	15	16 a	16 b	16 c	16 d	17
UDP	UTP	CMP	CDP	CTP	Principal hydrolysate analysis, 2-dim. technique				Hydrolysate control, 1-dim. technique

Fig. 29. — Two-dimensional chromatogram of the degradation of ribonucleic acid by Lactarius torminosus fungus enzyme in 24 hours. Included in the figure are one-dimensional control runs of the same hydrolysate and authentic 5'-ribonucleotides. The experimental procedure, solvents and concentrations of the spots applied in the analysis are similar to that in Fig. 26. The rf-values are given in Table 6.

No. of spot	1	2	3	4	5	6	7	8	9	10
Substance:	GTP	GDP	GMP	ITP	IDP	IMP	UTP	UDP	UMP	CTP

11	12	13	14	15	16 a	16 b	16 c	16 d	17
CDP	CTP	ATP	ADP	AMP	Principal hydrolysate analysis, 2-dim. technique				Hydrolysate control, 1-dim. technique

Fig. 30. — Two-dimensional chromatogram of the degradation of ribonucleic acid by Lactarius torminosus fungus enzyme in 24 hours. Included in the figure are one-dimensional control runs of the same hydrolysate and authentic 5-ribonucleotides. The experimental procedure, solvents and concentrations of the spots applied in the analysis are similar to that in Fig. 27. The rf-values are given in Table 6.

No. of spot	1	2	3	4	5	6	7	8	9	10
Substance:	GTP	GDP	GMP	ITP	IDP	IMP	UTP	UDP	UMP	CTP

11	12	13	14	15	16 a	16 b	16 c	16 d	17
CDP	CMP	ATP	ADP	AMP	Principal hydrolysate analysis 2-dim. technique				Hydrolysate control, 1-dim. technique

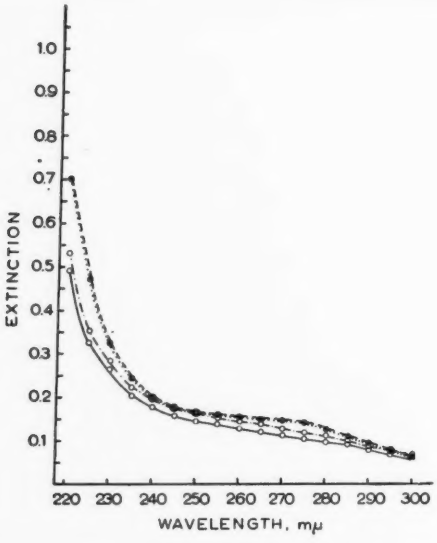

Fig. 33. — Absorption variations of blank eluates.

o-·-·-o-·-·-o Blank eluate corresponding to degradation product 7 a
o- - -o- - -o ,, ,, ,, ,, ,, ,, 7 b
o·····o·····o ,, ,, ,, ,, ,, ,, 7 c
o——o——o ,, ,, ,, ,, ,, ,, 7 d

fungus enzyme hydrolysate of ribonucleic acid after elution and neutralisation from a chromatographic paper. The paper in question was analysed with the solvent systems n-butanol — water in the first dimension and 0.1 M phosphate buffer pH 6.8 — ammonium sulphate — n-propanol in the second dimension. Fig. 33 shows the mean variations in blank absorptions analysed by the location of each degradation product according to the rf-value. All these chromatograms were also analysed by means of the same solvent systems. Moreover, the ultraviolet spectra of the hydrolysis products were measured separately from all the chromatographic papers analysed by all three solvent systems. Each degradation product, in all the cases, had a typical spectrum which corresponded accurately to the authentic ribonucleoside as anticipated from its rf-value. Ultraviolet spectrum determination thus confirmed the ribonucleoside nature of degradation end products.

The variations in the blank values proved surprisingly small when determined from material treated with n-butanol — wa-

ter and 0.1 M phosphate buffer pH 6.8 — ammonium sulphate — n-propanol solvents. The values in Fig. 33 are means of a total of 65 identical two-dimensional chromatographic runs given the above solvent treatment. Blank absorptions were generally greater and more difficult to control if material treated with the other solvent systems was used in the eluation. In spite of this it was possible to eluate typical nucleoside ultraviolet spectra from the analysed papers also with these solvents.

The absorption ratios at 250, 260 and 280 mμ for each compound isolated were also calculated from spectral data obtained in connection with identification studies by ultraviolet absorption measurements of hydrolysis end products (Fig. 25). The ratios were compared with values given in the literature for known ribonucleosides at pH 7.0 and with values obtained by the author from authentic samples. The calculated absorption

TABLE 7

ABSORPTION RATIOS AT 250, 260 AND 280 Mμ OF RIBONUCLEIC ACID HYDROLYSIS END PRODUCTS DIGESTED BY FUNGUS ENZYME COMPARED WITH KNOWN VALUES FOR AUTHENTIC RIBONUCLEOSIDES.

Compound	Absorbance Ratios	
	250/260	280/260
Authentic ribonucleosides:		
In the literature:		
Adenosine	0.79	0.15
Guanosine	1.18	0.68
Uridine	0.75	0.36
Cytidine	0.86	0.94
Obtained by the author:		
Adenosine	0.79	0.14
Guanosine	1.12	0.68
Uridine	0.75	0.36
Cytidine	0.86	0.93
Hydrolysis end products:		
(for remarks, see Figs. 25, 26 and 27)		
7 a	0.76	0.17
7 b	1.12	0.62
7 c	0.75	0.36
7 d	0.86	0.93

ratios of authentic pyrimidine ribonucleosides and related hydrolysis end products were in good agreement with known values reported in the literature. On the other hand, the calculated absorption ratios of authentic purine ribonucleosides and related hydrolysis end products varied somewhat from the ratios reported in the literature. The results are given in Table 7. In broad outline, however, they were all in agreement.

Phosphorus content of degradation products. — If the degradation products (spots 7a, 7b, 7c and 7d) shown in Figs. 25, 26 and 27, were ribonucleosides, as the rf-values and the ultraviolet spectrum measurements seemed to indicate, they should not contain any phosphorus.

The phosphorus content of the degradation products was studied with isotope ribonucleic acid labeled with P^{32} (RNA-P^{32}, Abbot Laboratories) as the substrate in the enzyme hydrolysis. The reaction mixture was similar to that used earlier. After 24 hours of digestion the hydrolysate was analysed by two-dimensional paper chromatography as before with all three solvent systems in the second dimension and in identical conditions with those of the previous analyses. The chromatograms were

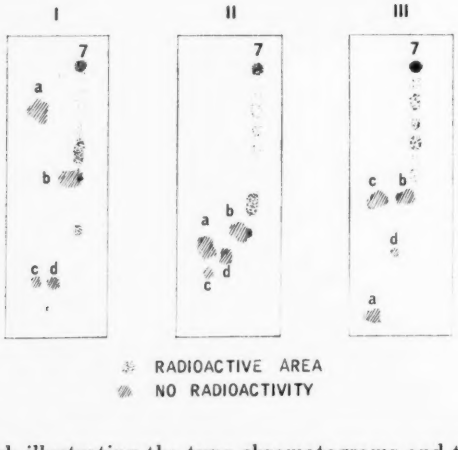

Fig. 34. — Graph illustrating the type chromatograms and the radioactivity distribution in degradation products of P^{32}-labeled ribonucleic acid degraded with Lactarius torminosus fungus enzyme. I, II, and III refer to the three different two-dimensional solvent systems described in detail in Table 6. The corresponding authentic chromatographic assays were presented in Figs. 25, 26 and 27.

developed in the same way by ultraviolet printing technique on photographic paper. The isotope substrate used was hydrolysed in exactly the same manner as the ordinary substrate used previously. After the localisation of the degradation products on the original chromatogram paper they were cut off and their radioactivity examined direct from the paper. There was no radioactivity in the area of the degradation products (spot 7a, 7b, 7c and 7d), which meant that they were compounds free from phosphorus. All radioactivity was concentrated at the starting point and in the unidentified degradation products which could not be moved with n-butanol — water solvent. Fig. 34 reveals the radioactive areas of each type of chromatogram and the areas free from radioactivity. Phosphorus determination agreed fully with the above identification studies.

To summarise the identification studies of hydrolysis products, since the rf-values and the ultraviolet spectrum findings agreed with those for the corresponding authentic ribonucleosides, and as the degradation products were phosphorus-free compounds, they were considered to be ribonucleosides liberated from ribonucleic acid under the influence of fungus enzyme.

QUANTITATIVE RIBONUCLEOSIDE DETERMINATION FROM HYDROLYSATE

Checking the isolation method with authentic samples. — The sole isolation method used was the two-dimensional paper chromatographic method. This is not a fully satisfactory method and includes sources of error. Hence it was checked and the magnitude of the error factors assessed before the actual analysis. 5, 10, 15 and 20 μl of 0.5 per cent solution of known nucleosides (Pabst Laboratories) buffered to pH 7.0 were pipetted according to the schedule already used and as in the hydrolysate studies onto Whatman No. 1 paper for a two-dimensional run. The first dimension was run for 36 hours at +20 °C with n-butanol — water solvent. The second dimension was then run for 27 hours at +27 °C with 0.1 M phosphate buffer pH 6.8 — ammonium sulphate — n-propanol solvent. The spots were localised by ultraviolet printing technique on photographic paper and from it on the chromatogram sheet itself. The spots were cut off

carefully, cut up and eluated by shaking mechanically for 2 hours at +37 °C in 0.1 N HCl. A piece of paper only of corresponding size was cut from the immediate vicinity of each spot and from the same rf-value range. These pieces were treated in the same way as the actual spots to obtain the blank values needed for correction of the extinction readings. The concentration of nucleosides in the eluate was determined spectrophotometrically from the maximum absorption range of each by means of certain known constants. The mean blank value employed in the extinction corrections was the same as was used in the analyses described earlier (mean result of 65 determinations shown in Fig. 33).

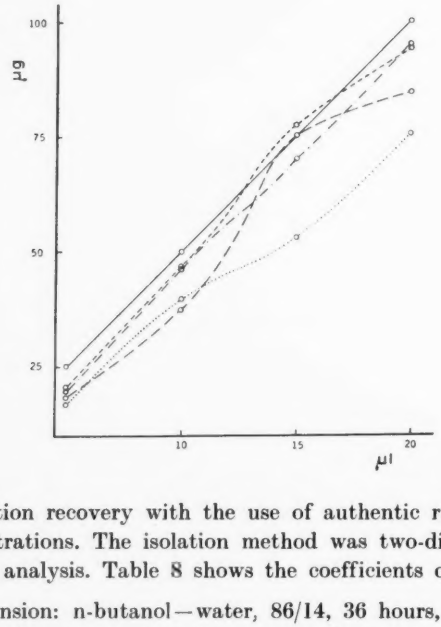

Fig. 35. — Elution recovery with the use of authentic ribonucleosides in different concentrations. The isolation method was two-dimensional paper chromatography analysis. Table 8 shows the coefficients of error.

Solvents: 1-dimension: n-butanol—water, 86/14, 36 hours, at +20 °C with the ascending technique. 2-dimension: 0.1 M phosphate buffer pH 6.8—ammonium sulphate—n-propanol, 100/60/2, 14 hours, at +27 °C with the ascending technique. The range of nucleoside concentrations at the starting points was 25—100 µg.

o——o——o Initial amount
o·····o·····o Elution yield from adenosine in different concentrations
o— —o— —o Elution yield from guanosine in different concentrations
o—·—·—o—·—·—o Elution yield from uridine in different concentrations
o— – —o— – —o Elution yield from cytidine in different concentrations

TABLE 8

ISOLATION OF AUTHENTIC RIBONUCLEOSIDES BY THE TWO-DIMENSIONAL PAPER CHROMATOGRAPHY TECHNIQUE. SOLVENTS: IN THE FIRST DIMENSION N-BUTANOL — WATER, 86/14, ASCENDING TECHNIQUE, 36 HRS, +20 °C; IN THE SECOND DIMENSION 0.1 M PHOSPHATE BUFFER PH 6.8 — AMMONIUM SULPHATE — N-PROPANOL, 100/60/2, ASCENDING TECHNIQUE, 14 HRS, +27 °C.

Authentic Stock Solution 0.5 %		Recovery							
		Adenosine		Guanosine		Uridine		Cytidine	
µl	µg	Yield µg	Coeff. of Error	Yield µg	Coeff. of Error	Yield µg	Coeff. of Error	Yield µg	Coeff. of Error
5	25	16.8	1.49	18.6	1.34	20.0	1.25	20.5	1.22
10	50	39.7	1.26	37.2	1.34	46.7	1.07	46.7	1.07
15	75	53.0	1.42	75.0	1.00	70.0	1.07	77.6	0.97
20	100	75.5	1.32	84.5	1.18	95.0	1.05	94.4	1.06

The variations in the elution yields of the different nucleosides in four different concentrations are shown in Fig. 35. It will be noted that it was possible to recover almost without loss the pyrimidine nucleosides most readily soluble. On the other hand, the purine nucleosides with poor solubility were difficult to isolate and the losses were great. Table 8 gives the calculated coefficients of error arising from the technique used, for all the nucleosides in different concentrations.

Quantitative isolation of the liberated ribonucleosides. — 24-hour old fungus enzyme hydrolysate of the same composition as the reaction mixture employed earlier was used for the analyses. The sample quantity of 75 µl was arrived at after preliminary tests which showed that this amount of hydrolysate gave eluate extinction values for the degradation products that corresponded best to the calibration values. Ensuring accurate calibration, six 75 µl batches of hydrolysate were analysed simultaneously by the corresponding solvent systems, i.e. n-butanol — water in the first dimension and 0.1 M phosphate buffer pH 6.8 — ammonium sulphate — n-propanol in the second dimension. The identical ascending two-dimensional chromatographic technique was applied in the analyses. The run times were the

TABLE 9

RIBONUCLEOSIDES IN PERCENTAGE MOLAR PROPORTIONS LIBERATED FROM RIBONUCLEIC ACID UNDER THE INFLUENCE OF LACTARIUS TORMINOSUS FUNGUS ENZYME. FINAL PH OF THE REACTION MIXTURE 4.0. REACTION TIME 24 HRS. TEMPERATURE +37 °C. RIBONUCLEOSIDE HYDROLYSIS DEGREE 45.3 PER CENT OF RNA.

Reaction Mixture	Purine Nucleosides			Pyrimidine Nucleosides			6-amino Compounds	6-keto Compounds
	Adenosine	Guanosine	Total	Uridine	Cytidine	Total		
RNA, Light & Co., Ltd., twice purified, 20 mg/ml, pH 3.85, 2 ml Lactarius torminosus lyophilisate, 20 mg/ml, pH 5.0, 2 ml	32.5	35.7	68.2	12.7	19.0	31.7	51.5	48.4

same. The spot development, localisation, cutting off, cutting up and eluation methods were the same. The extinction values were determined from the eluates or their dilutions against the solution employed for elution, and the values were corrected with the mean blank value and coefficients of error calculated separately for the purpose. The concentrations of nucleosides in the spots were then calculated from the corrected extinction values with aid of known constants. With these concentrations known it was possible to determine the percentual degree of nucleoside hydrolysis of the substrate. Finally, the total quantity of each liberated nucleoside was calculated in percentage molar proportions.

Table 9 gives the results of the quantitative isolation of liberated ribonucleosides from ribonucleic acid digested by Lactarius torminosus fungus enzyme in the course of 24 hours at +37 °C and pH 4.0.

DISCUSSION

The degradation products of ribonucleic acid digested by fungus ribonucleolytic enzymes may be divided into two main groups: inorganic phosphorus and nucleic acid derivatives.

Of the many phosphorus determination methods available, the microtechnique modified by Berenblum and Chain was selected first and foremost for its reliability although it is perhaps a laborious and time consuming method which requires careful calibration. The method of determining radioactive phosphorus described by Roth and Milstein was adopted as a control. The diminutive amount of isotope ribonucleic acid and its low radioactivity made it impossible to calibrate the two methods to correspond. However, the phosphorus-liberating phenomenon was shown up qualitatively even when isotope substrate was used. All the quantitative inorganic phosphorus determinations are in fact based solely on the ordinary phosphorus determinations. They proved reliable and could be repeated.

Optical and paper chromatographic methods lend themselves very readily to the examination of nucleic acid derivatives originating as hydrolysis products. They are simple, specific and easy to control. The circular (84) recently published by Pabst Laboratories of the spectrophotometric and chromatographic constants of ribonucleic acid derivatives and of suitable investigation methods was very useful. The methods were found to be very reliable since they could be used in combination and were thus easy to check.

The results show that under the influence of the fungus enzymes used ribonucleic acid is depolymerised very rapidly into acid-soluble form. This hydrolysis phase can be followed by various optical methods, e.g. following the decrease in extinction at 300 mμ and the increase in extinction at 260 mμ caused by acid soluble degradation products. Or it can be followed turbidimetrically as a fall in transmission values at 420 mμ. It was more difficult, however, to follow the depolymerisation phase chromatographically. This is an extremely rapid phase and occurs at the very start of the reaction. It is, moreover, obviously a transient phenomenon.

The attempt to register chromatographically with several sol-

vent systems the mononucleotides produced by depolymerisation met with poor success. This was due to the very early cleavage of inorganic phosphorus as the enzyme hydrolysis progressed, leaving only the possibility that nucleosides were demonstrable. This inorganic phosphorus-liberating velocity was found to be somewhat faster from isotope than from ordinary substrate. It is, however, difficult to state whether these two different methods with different substrates are comparable. However, the substrate turned acid-soluble at a phase so much earlier than the beginning of the liberation of inorganic phosphorus that there was reason to suspect the occurrence of an acid soluble mono- or oligonucleotide intermediate phase in the reaction mixture. Hence the enzyme hydrolysis was also observed manometrically in bicarbonate buffer when CO_2 should be liberated in the presence of acid nucleotides. The CO_2 could then be registered manometrically in Warburg's apparatus. No liberation of CO_2, however, occurred in the time taken by the substrate to become acid-soluble and for the start of inorganic phosphorus liberation. Moreover, the point was controlled chromatographically. The two-dimensional chromatographic system was calibrated carefully with authentic 5'-ribonucleotides The samples were taken from the reaction mixture at different stages of hydrolysis and analysed in identical conditions. However, it was not possible to demonstrate in the hydrolysis products any nucleotides comparable with the controls. It was obvious from this that nucleotides originating as intermediate products are hydrolysed further very rapidly to the nucleoside stage.

The vigorous liberation of inorganic phosphorus from the reaction mixture with the progress of hydrolysis supports the above finding. When Armillariella mellea lyophilisate was employed as the enzyme in the reaction mixture it was possible to change the phosphorus content of nearly the entire substrate to inorganic form within 75 hours in standard conditions. The liberation of inorganic phosphorus is closely associated with the activity of the enzyme and its concentration in the reaction mixture, as was shown in the experiments with various concentrations of active and inactive enzymes. No spontaneus liberation of inorganic phosphorus was observed.

Thus, the occurrence of nucleosides in the hydrolysate as reaction end products is possible. The identification of the end products showed that this actually occurred. 45.3 per cent of the substrate was hydrolysed to the nucleoside stage in optimal conditions during a 24-hour reaction period. The chromatographic picture of the hydrolysate with different solvents was very consistent. Purine nucleosides occurred in the hydrolysate at perhaps a slightly earlier phase than pyrimidine nucleosides, though both were liberated very rapidly from the substrate. No fungus enzyme resistant core formation was found. On the contrary, the substrate became acid-soluble in its entirety.

The errors occurring in the quantitative determinations of reaction products were eliminated as far as possible by performing the experiments in carefully calibrated test conditions and by checking the error limits of the isolation and measuring methods against known components prior to the actual analysis. It was thus possible to use coefficients of error and constants for the final calculations and achieve more reliable results.

The digestion of ribonucleic acid under the influence of the fungus enzymes used may be summarised briefly as follows:

$$\text{RNA} \xrightarrow{\text{Depolymerase}} \begin{cases} \text{Nucleotides} \\ \text{(mono-,oligo-,)} \\ \text{acid-soluble} \end{cases} \xrightarrow{\text{Phosphatase}} \begin{cases} \text{Inorganic P} \\ \text{Nucleosides} \end{cases}$$

ATTEMPTS TO PURIFY THE TWO RIBONUCLEOLYTIC FUNGUS ENZYMES

METHODS AND MATERIAL

Methods in general. — The analytical methods used were phosphorus and nitrogen analyses, fresh (air-dried) weight and dry weight determinations, paper electrophoresis and salt fractionation, as described above.

The measurement of ribonuclease activity was performed by Kunitz' method in which the decrease caused by the enzyme in the extinction of substrate at 300 mμ, pH 5.0 and at +25 °C is determined spectrophotometrically.

Enzymes. — Of the fungus enzymes, Armillariella mellea and Lactarius torminosus were selected as the objects of investigation. Both have good ribonuclease activity (93 and 84 per cent) although not the highest for the raw material. The activity analyses were made at the different phases of purification from the following intermediate products: (1) 1 : 10 water extract of crude fungus powder, (2) lyophilisates of crude fungus extracts which have undergone additional electrophoretic purification, and (3) lyophilisate fractions precipitated with $(NH_4)_2SO_4$ in different degrees of saturation. The activity of the different enzyme preparations was finally calculated in Kunitz units.

Crude fungus powder served as the primary material for the purification of both Armillariella mellea and Lactarius torminosus. It was finely crushed, air-dried and dust-like fungus powder, and its activity remained unchanged during the 4 years of the investigation. Table 11 gives the dry weights and nitrogen contents of the preparations.

Crude fungus extract: The preparations were suspended separately in distilled water in the ratio 1 : 10. The suspension was incubated at + 37 °C for 2 hours and the insoluble part centri-

fuged off. The filtrate was drawn rapidly by suction through Whatman No. 1 filter paper. The final filtrate was a strongly coloured, turbid and slightly viscose solution. Both filtrates were drawn rapidly by vacuum suction several times through Chamberlain porcelain filters (pore size 5L5). On the first few occasions a thick oil-like deposit was precipitated on the filter surface. This diminished after a time. The solution finally clarified but was still strongly coloured. Table 11 gives the results of the dry-weight determinations of the extracts and their nitrogen contents.

Lyophilisation of the filtrates purified by Chamberlain filtration was performed at —80 °C and in a vacuum of 0.05 mmHg. After 24 hours a fine powder-like substance was obtained which was extremely hygroscopic. Freeze-drying fails if Chamberlain filtration is insufficient, gives only an oily paste difficult to handle.

Crystalline pancreatic ribonuclease was used only for the calibration of Kunitz' spectrophotometric method. The concentration of the stock solution was 1 mg of enzyme in 100 ml of distilled water containing 0.1 per cent gelatin. The working solutions were obtained by diluting the stock solution.

The substrate used was commercial ribonucleic acid (British Drug Houses, Ltd., London) as such. The concentration of the substrate stock solution was 0.1 per cent in 0.1 M acetate buffer, pH 5.0. In the spectrophotometric method of Kunitz the final concentration of the substrate was 0.05 per cent. The final pH of the reaction mixture was checked by control measurements and when necessary adjusted with 0.1 N HCl.

EXPERIMENTAL PROCEDURE AND RESULTS

PURIFICATION OF THE FUNGUS ENZYMES

Fungus enzymes preserved in lyophilised form were purified further mainly by paper electrophoresis and by $(NH_4)_2SO_4$ fractional precipitations.

Paper electrophoresis. — As lyophilisates, even when several times freeze-dried, were difficult to store dry, the lyophilisated preparations were purified further by semi-preparative paper elec-

trophoresis. The voltage range of the power unit used was 0—400 V, and current strength 0—12 mA. The electrolyte used was 0.05 N medinal-veronal buffer, pH 8.6. A concentrated aqueous solution of the enzyme was pipetted rapidly on paper dipped in electrolyte liquid and the current switched on. With 200 V and ca. 10 mA and a run time of 2—3 hours a distinct anode fraction was obtained and the enzyme activity accompanied it. The fraction was strongly fluorescent in ultraviolet light and therefore easy to localise and cut off. The fraction was eluated into distilled water, incubation time 1 hour at +37 °C. The eluate was dialysed against distilled water at +5 °C for 24 hours, changing the water frequently. After the dialysis the eluate was freeze-dried in the conditions described above. After 24 hours of freeze-drying a fine amorphous powder was obtained which was much lighter in colour than the previous lyophilisate and had equally good keeping qualities in dry form. Paper electrophoresis with its extraction, elution and repeat freeze-drying phases is not a necessary measure but rather a precaution necessary for the preservation of the material. In electrophoretic purification the activity of the lyophilisates remains unchanged or increases slightly.

Fractional precipitations with $(NH_4)_2SO_4$. — Precipitations were performed to increase the activity of the enzymes. Turbidimetric titration of the lyophilised preparations of both fungi was performed at the different $(NH_4)_2SO_4$ saturation phases to find suitable fraction limits. Fig. 36 gives an idea of the precipitation of enzyme protein: it shows the transmission values of 0.2 per cent Armillariella mellea and 1.0 per cent Lactarius torminosus as a function of the degree of $(NH_4)_2SO_4$ saturation. The amount of dry substance (mg/ml) proved to be the same in the above-mentioned solutions if their $(NH_4)_2SO_4$ saturation degree was raised rapidly to 100 per cent. On the basis of the results 3-phase fractionation was decided on. An endeavour was made to separate the first fraction in a 0—0.40 saturation of $(NH_4)_2SO_4$, the second in a 0.40—0.60 saturation of $(NH_4)_2SO_4$ and the third in a 0.60—1.00 saturation of $(NH_4)_2SO_4$.

25 ml of aqueous solution of 20 per cent fungus lyophilisate was used for the fractionation. The $(NH_4)_2SO_4$ was always added in an accurately measured volume of filtrate of the preceding precipitate. Precipitation was performed at +6 °C. The precipita-

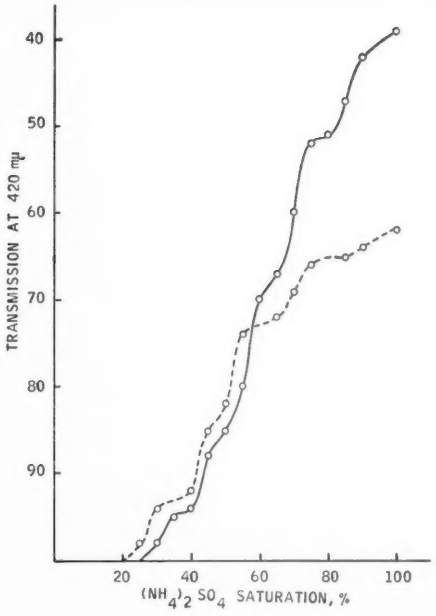

Fig. 36. — Precipitation of protein from the solutions containing lyophilised fungus enzymes in different saturations of $(NH_4)_2SO_4$. Both solutions contained the same amount of dry substance calculated per ml.

1.0 per cent Lactarius torminosus solution

0.2 per cent Armillariella mellea solution

tes were separated by centrifugation, washed 3 times with the corresponding $(NH_4)_2SO_4$ solution and finally suspended in 5 ml of distilled water and dialysed similarly to the corresponding filtrate samples against distilled water. After dialysis they were made up with distilled water to a volume of 10 ml. For the calculation of dry weights, 1 ml of the solution diluted as described above was evaporated on a watch glass and dried at +105—110 °C for 6 hours. 0-controls were performed from the same volumes and in identical conditions with distilled water.

Fractionation of the lyophilisate of Armillariella mellea: 5 g of the lyophilisate was dissolved in 25 ml of distilled water. The $(NH_4)_2SO_4$ content of the solution was raised carefully to the saturation degree 0.40. No precipitate was formed. At the 0.60 degree of $(NH_4)_2SO_4$ saturation a small precipitate was obtained and recovered. $(NH_4)_2SO_4$ crystals were added to the

filtrate until the degree of saturation was 1.0. A new, slight precipitate was obtained and recovered. Table 12 gives the quantitative amounts of the isolated fractions and their nitrogen content. The final weights of the fractions were corrected for measuring losses so as to correspond to the original amount of primary material. The fractions and the last filtrate were kept for activity analyses.

Fractionation of the lyophilisate of Lactarius torminosus: 5 g of the lyophilisate was dissolved in 25 ml of distilled water. The $(NH_4)_2SO_4$ content of the solution was raised carefully to the 0.40 degree of saturation. The small opalising precipitate that was formed in 2 hours was too small to separate. At the 0.60 degree of $(NH_4)_2SO_4$ saturation a distinct precipitate was obtained in 2 hours and isolated. $(NH_4)_2SO_4$ crystals were added to the filtrate until the degree of saturation was 1.0. The opalising precipitate that formed was again too small to separate. Table 12 gives the quantitative amounts of the fraction corrected to equal the original quantity of primary material. The fraction and the last filtrate were kept for activity analyses.

SPECIFIC ACTIVITY OF CRYSTALLINE PANCREATIC RIBONUCLEASE

Before commencing the analysis of the activities of various fungus enzyme preparations, the spectrophotometric method of Kunitz was calibrated by determining the specific activity of crystalline pancreatic ribonuclease from a commercial preparation.

The following solutions, pre-heated at +25 °C room temperature, were pipetted into quartz cells 1 cm in diameter.

		Test:	*Blank:*	*Control:*
Substrate:	RNA, 1 mg/ml, pH 5.0	2 ml	—	2 ml
Enzyme:	0.002 mg/ml in 0.1 % gelatin	2 „	2 ml	—
Buffer:	0.1 M acetate buffer, pH 5.0	—	2 „	2 „

Before pipetting the reaction mixture into the cells, the spectrophotometer was calibrated by measuring the extinction (E_o) at 300 mμ of the control solution at the beginning of the reaction against the blank solution. After the enzyme was added to the substrate the decrease in the extinction of the substrate

was measured at 300 mμ for 10 minutes at 1-min. intervals against blank solution. The cells were then allowed to stand at room temperature until the lowest final reading of the extinction (E_f) at the termination of the reaction. The decrease in the extinction values as a function of the reaction time (E *versus* *t*) and as a logarithmic value in regard to the reaction time [log(E—E_f) *versus* *t*] were calculated. The results are shown in the form of a graph where the linear decrease in extinction is illustrated in both cases by a straight line. The slope of this

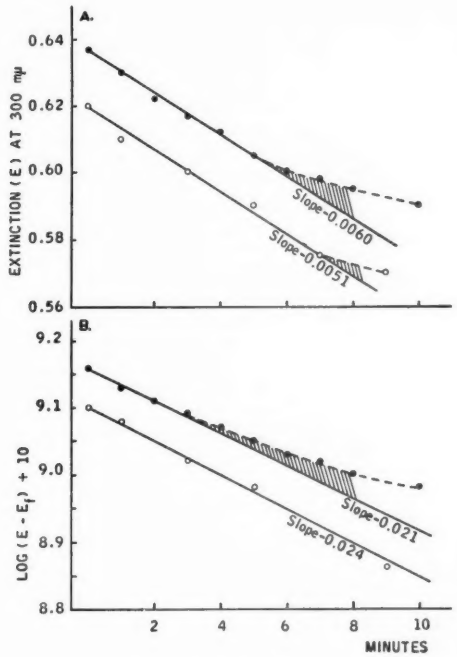

Fig. 37 a and 37 b. — The effect of crystalline pancreatic ribonuclease on the extinction of 0.05 per cent solution of ribonucleic acid at 300 μm. Digestion mixture: RNA (British Drug Houses, Ltd.), 1 mg/ml, 2.0 ml. PNRase (Worthington Biochemical Corporation), 0.002 mg/ml, 2.0 ml. Final digestion mixture, pH 5.0, in acetate buffer 0.05 M. Temperature +25 °C.
The specific activity of the crystalline pancreatic ribonuclease used, given in Table 10, was calculated from the decrease in extinction.

●——●——● Slope — 0.0060 and — 0.021: crystalline pancreatic ribonuclease, conc. 1 μg/ml.

○——○——○ Slope — 0.0051 and — 0.024: crystalline pancreatic ribonuclease, conc. 1 μg/ml.

TABLE 10

EFFECT OF RIBONUCLEASE ON ULTRAVIOLET LIGHT ABSORPTION AT 300 mμ OF YEAST NUCLEIC ACID.

DIGESTION MIXTURE: RNA (BRITISH DRUG HOUSES, LTD.) 1 MG/ML, 2.0 ML. CRYSTALLINE PANCREATIC RIBONUCLEASE (WORTHINGTON BIOCHEMICAL CORP.) 0.002 MG/ML (IN 0.1 % GELATIN), 2.0 ML. FINAL DIGESTION MIXTURE, PH 5.0, IN ACETATE BUFFER 0.05 M.

Number of Experiment	Yeast Nucleic Acid	E_o	E_f	I			II		
				Slope of log $(E-E_f)$ *versus t*	Activity KU per ml	Activity KU per mg Ribonuclease	Slope of E *versus t*	Activity KU per ml	Activity KU per mg Ribonuclease
1.	British Drug Houses, Ltd.	0.637	0.494	-0.021	0.048	48	-0.0060	0.042	42
2.	—„—	0.620	0.495	-0.024	0.055	55	-0.0051	0.041	41

line can be calculated from the linear decrease in extinction per time unit. The enzyme activity per ml of enzyme solution can be calculated in the former case (E *versus t*) by dividing the value of a known slope by the difference (E_o-E_f) and in the latter case [log $(E-E_f)$ *versus t*] by multiplying the logarithmic value of the slope by —2.3. By dividing the activity values of the enzyme per ml by the enzyme concentration in this volume the activity can be expressed in mg per crystalline enzyme.

A unit of ribonuclease activity is defined as the amount of enzyme which is capable of causing a decrease of 100 per cent per minute in the E_o-E_f of the substrate at 25 °C and pH 5.0. The standard of unit activity is termed Kunitz Unit (KU).

Figs. 37a and 37b show the results of the double determinations of the decrease in the extinction of the substrate during ribonuclease digestion. The measurements were made at 300 mμ in the standard conditions described in the foregoing. The concentration of the enzyme was 1 μg/ml. Fig. 37a expresses the decrease in substrate extinction as a direct function of the reaction time. Fig. 37b gives the decrease in extinction logarithmically.

Table 10 gives the numerical values of both calculations of the double determinations. The mean activity of crystalline pancreatic ribonuclease was found to be 46.5 KU per mg.

SPECIFIC ACTIVITY OF FUNGUS ENZYMES AT DIFFERENT PHASES OF PURIFICATION

The activity was measured by Kunitz' spectrophotometric method. The substrate concentration of the reaction mixture, its temperature, ion concentration and pH were kept constant and identical with the conditions in which the activity of crystalline pancreatic ribonuclease was tested. The diameter of the cells used in the measurements was the same. All the components of the reaction mixture pipetted into the cells with the exception of the enzymes were in the same volume and concentration ratio as for the crystalline ribonuclease test. Fungus enzyme preparations representing the following different degrees of purification were used: (1) 1 : 10 aqueous extract of crude fungus powder, (2) lyophilisates of crude fungus extracts which have undergone additional electrophoretic purification and (3) $(NH_4)_2SO_4$ fractions of the lyophilisates at various degrees of saturation. All the preparations mentioned above, representing a different degree of purity and different enzyme activity, were made from both Armillariella mellea and Lactarius torminosus. Dry-weight analyses were made and the nitrogen content determined for each preparation. The concentration of the fungus enzyme in the reaction mixture was as far as possible kept at 0.1 mg/ml calculated by dry weight. The enzyme concentration of the reaction mixture in relation to nitrogen (μg N/ml) was determined parallelly. It was possible to use such a standard concentration or its approximate only for dry fungus powder or lyophilisate as their enzyme activities did not differ from one another to any appreciable extent. The concentration of the enzyme fractions in the reaction mixture, obtained by $(NH_4)_2SO_4$ precipitation, was determined by their activity. The most advantageous procedure was to use the $(NH_4)_2SO_4$ fractions in the reaction mixture in a dilution that represented the amount of 0.5 per cent unfractionated enzyme dilution remaining in the fraction in question. The dilutions were prepared on this basis consistently in all the analyses. The activity of each enzyme preparation was expressed in Kunitz units per ml of the reaction mixture, per dry weight (mg) of the enzyme, or in terms of the nitrogen (mg N) content of the enzyme.

Fig. 38 and Table 11 give the ribonuclease activities, determ-

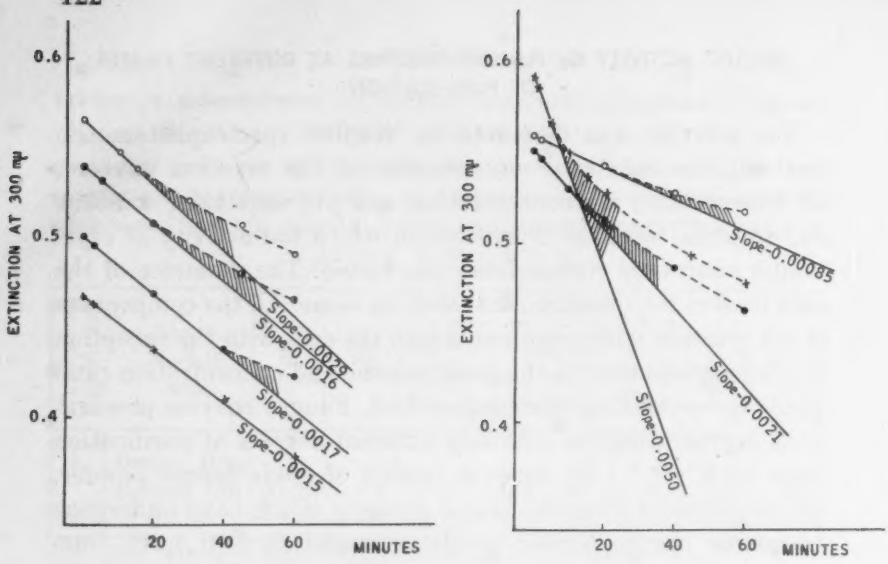

Fig. 38. — The effect of crude fungus extracts and their lyophilisates purified further by electrophoresis on the extinction of 0.05 per cent solution of ribonucleic acid at 300 mμ. Digestion mixture: RNA (British Drug Houses, Ltd.), 1 mg/ml, 2.0 ml. Fungus crude extract or its purified lyophilisate, 2.0 ml, in the concentrations mentioned below. Final digestion mixture, pH 5.0, in Michaelis buffer 0.023 M. Temperature +25 °C. The specific activities of the fungus enzyme preparations used, given in Table 11, were calculated from the decrease in extinction.

- Slope — 0.0017: Armillariella mellea crude extract, conc. 1 mg dry substance/ml.
- Slope — 0.0029: Armillariella mellea lyophilisate purified further by electrophoresis, conc. 0.87 mg dry substance/ml.
- Slope — 0.0015: Lactarius torminosus crude extract, conc. 2 mg dry substance/ml.
- Slope — 0.0016: Lactarius torminosus lyophilisate purified further by electrophoresis, conc. 0.88 mg dry substance/ml. (The concentrations refer to the final fungus enzyme concentration of the reaction mixture.)

Fig. 39. — The effect of fungus enzyme preparations fractionated by $(NH_4)_2SO_4$ on the extinction of 0.05 per cent solution of ribonucleic acid at 300 mμ. Digestion mixture: RNA (British Drug Houses, Ltd.), 1 mg/ml, 2.0 ml. Fractionated fungus enzymes, 2.0 ml, in the concentrations, mentioned below. Final digestion mixture, pH 5.0, in Michaelis buffer 0.023 M. Temperature +25 °C. The specific activities of the fungus enzyme fractions used, given in Table 12, were calculated from the decrease in extinction.

- Slope — 0.00085: Armillariella mellea, fraction I+II, conc. 0.073 mg dry substance/ml.
- Slope — 0.0021: Armillariella mellea, fraction III, conc. 0.047 mg dry substance/ml.
- Slope — 0.0050: Lactarius torminosus, fraction II, conc. 0.019 mg dry substance/ml.

TABLE II

ANALYTICAL VALUES OF THE RIBONUCLEOLYTIC ACTIVITY OF FUNGUS ENZYME PREPARATIONS.

Preparation	Fungus	Dry Weight %	N %	Concentration in Digestion Mixture		Slope of E *versus t*	Activity		
				mg Dry Substance per ml	µg N per ml		KU per ml	KU per mg Dry Substance	KU per mg N
Crude fungus powder	Armillariella mellea	94.0 ± 0.30	3.14 ± 0.06	—	—	—	—	0.0100	0.31
	Lactarius torminosus	95.1 ± 0.10	2.73 ± 0.27	—	—	—	—	0.0042	0.15
Fungus extract 1 : 10	Armillariella mellea	50.2 mg/ml	4.37 ± 0.16	1.00	43.9	—0.0017	0.0113	0.0113	0.25
	Lactarius torminosus	37.1 mg/ml	3.14 ± 0.05	2.23	67.0	—0.0015	0.0095	0.0043	0.14
Lyophilisate purified further by electrophoresis	Armillariella mellea	87.23 ± 0.33	3.47 ± 0.09	0.87	30.3	—0.0029	0.0143	0.0160	0.47
	Lactarius torminosus	88.26 ± 0.20	3.57 ± 0.15	0.88	31.5	—0.0016	0.0087	0.0099	0.28
Most active $(NH_4)_2SO_4$—fractions	Armillariella mellea	(see Table 12.)	8.92 ± 0.20	0.047	4.2	—0.0021	0.0110	0.23	2.60
	Lactarius torminosus		7.90 ± 0.80	0.019	1.6	—0.0050	0.0275	1.40	18.00

ined by the Kunitz' spectrophotometric method, of dry fungus powder, its aqueous extract (1 : 10) and lyophilisate prepared from the extract and purified further by electrophoresis. The substrate used was 0.05 per cent ribonucleic acid in standard conditions. The table also gives the analytical values of different enzyme preparations and the enzyme concentrations of the reaction mixture in terms of both dry weight and nitrogen. The activity determinations were made with all their intermediate phases and the final enzyme activity of each preparation was expressed in Kunitz units per mg dry weight and per mg N. The activity figures assigned to crude fungus powder were theoretical, given principally to obtain starting values. They were calculated from measuring results obtained with 1 : 10 aqueous extracts of crude fungus powder. The activity figures obtained for actual fungus extracts were naturally of the same

TABLE 12

PURIFICATION OF LYOPHILISED FUNGUS ENZYME PREPARATIONS BY FRACTIONAL PRECIPITATION WITH $(NH_4)_2SO_4$.

Fungus	Lyophilisate		Fractional Precipitation				Concentration in Digestion Mixture		Slope of E *versus t*	Activity			Yield	
	Quantity to be fractionated, g	Original Total Activity, KU	Degree of $(NH_4)_2SO_4$ Saturation	Name of Fraction	Yield, mg Dry Substance	N %	mg Dry Substance per ml	μg N per ml		KU per ml	KU per mg Dry Substance	KU per mg N	Total Activity, KU	Recovery, %
Armillariella mellea	5.0	70.0	0.00 — 0.60	I + II	7.3	7.46 ± 0.01	0.073	5.4	—0.00085	0.0045	0.062	0.83	0.5	
			0.60 — 1.00	III	5.0	8.92 ± 0.20	0.047	4.2	—0.0021	0.0110	0.23	2.60	1.2	
			(filtrate)		(75 ml)	—	(1 : 2)	—	—0.0032	0.0190	—	—	2.9	
													4.6	6
Lactarius torminosus	5.0	44.0	0.40 — 0.60	II	24.0	7.90 ± 0.80	0.019	1.6	—0.0050	0.0275	1.4	18.0	34	
			(filtrate)		(75 ml)	—	(1 : 2)	—	—0.0020	0.0114	—	—	2	
													36	80

magnitude. The activity figures in Kunitz units per mg N of the preparation show that activities were generally very slight at this phase and that the enzyme activity of Lactarius torminosus was only half of that of Armillariella mellea. The activities of lyophilisates made from fungus extracts and purified further electrophoretically were double the former, but the activity of Lactarius torminosus was still roughly a half of that of Armillariella mellea. Only $(NH_4)_2SO_4$ fractionation produced a more effective enhancement of enzyme activity. True the salt fractionation of Armillariella mellea did not succeed as well as that of Lactarius torminosus, but even so enzyme activity increased approximately tenfold in the best fraction (fraction III). Salt fractionation of Lactarius torminosus succeeded better and the enzyme activity of the best fraction (fraction II) increased 120 times compared with the starting value. Lactarius torminosus proved much better at this phase than Armillariella mellea, though its initial activity was smaller.

Fig. 39 and Table 12 show the ribonuclease activities, determined by the spectrophotometric method of Kunitz, of enzyme preparations fractionated at the different degrees of $(NH_4)_2SO_4$ saturation. The table gives the quantity (g) of the lyophilisate employed as the primary material and its calculated total activity (KU). The quantitative amounts of the fractions precipitated at different degrees of $(NH_4)_2SO_4$ saturation are presented as the direct results of dry weight analysis corrected for measuring losses to correspond to the original quantities of primary material. On the basis of the analyses of the fractions, the enzyme concentration in the reaction mixture was given in mg of dry substance per ml and in terms of nitrogen content (μg N/ml). The activity determinations are given in Table 12 with all their intermediate phases and the final enzyme activity of each fraction is expressed in Kunitz units per mg of dry matter and per mg N. Finally, the sum of the ribonuclease activities of all fractions isolated by salt fractionation and of the last filtrate was calculated in per cent of the original total activity of the lyophilisate. The results show that the salt fractionation of Armillariella mellea had little success as only 6 per cent of the original activity was isolated. Lactarius torminosus salt fractionation succeeded well and 80 per

TABLE 13

ACTIVITIES OF LACTARIUS TORMINOSUS FUNGUS ENZYME PREPARATIONS AND CRYSTALLINE PANCREATIC RIBONUCLEASE, AND THEIR COMPARISON.

Enzyme Preparations	Activity		Relative Activity	
	KU per mg Dry Substance	KU per mg N	KU per mg Dry Substance	KU per mg N
Lactarius torminosus: Primary material (crude fungus material)	0.0042	0.15	1	1
Final product (fraction II, isolated in ammonium sulphate saturation 0.40—0.60)	1.40	18.00	333	120
Crystalline pancreatic ribonuclease: 1 × cryst., salt free. Worthington Biochemical Corp. Freehold, New Jersey.	46.5	—	c. 10000	—

cent of the original activity was isolated; 95 per cent of the isolated activity was recovered in precipitate form (fraction II).

Table 13 compares the activity of the raw material of Lactarius torminosus fungus enzyme with the activity of the best fraction of the same fungus obtained by $(NH_4)_2SO_4$ precipitation and with the activity of crystalline pancreatic ribonuclease. Coefficients representing the increase in activity in terms of both dry weight and nitrogen content were determined for the best fungus enzyme fraction. These coefficients show that the activity of the best fraction was some 30 times weaker than that of crystalline pancreatic ribonuclease.

DISCUSSION

In the purification of various fungus enzyme preparations by $(NH_4)_2SO_4$ precipitation the activity analyses of the fractions were made by the spectrophotometric method of Kunitz. To assess the reliability of the method it was first calibrated with crystalline pancreatic ribonuclease. The concentration of

crystalline ribonuclease in the reaction mixture was varied from 0.005 to 0.00005 mg/ml. The results varied with the different enzyme concentrations and to some extent also with the method of calculation. If the enzyme concentration in the reaction mixture was 0.00005 mg/ml or less no change was found in extinction. When high enzyme concentrations were used the results of parallel determinations changed considerably even due to the technical difficulty of following a rapid reaction. The results of parallel determinations were consistent and reliable when the ribonuclease concentration in the reaction mixture was 0.001 mg/ml. This concentration was therefore employed for calculation of the specific activity of the enzyme.

Similar observations on the sensitiveness and reliability of the method were made with fungus enzymes. The greatest errors of measurement were due to either too slow or too rapid a reaction. The reliability of the slope of the graph suffered from the failure to make the first three readings linear. This requirement must be complied with before the results can be interpreted in detail. It makes the registration of weak enzyme activities very laborious. The activity measurements of fungus enzyme fractions isolated by $(NH_4)_2SO_4$ precipitation, on the other hand, were easier to perform as it was possible to regulate the velocity of the reaction better. However, a host of preliminary tests were necessary to obtain the correct enzyme concentration for the reaction mixture. The concentration of Lactarius torminosus fraction precipitated at an $(NH_4)_2SO_4$ saturation degree of 0.40–0.60 was varied in the reaction mixture between the limits 0.5–0.005 mg/ml. Maximum activity was registered in the reaction mixture with an enzyme concentration of c. 0.02 mg/ml (Table 12).

A drawback of the spectrophotometric method was that the reaction was fixed at pH 5. This is not the optimal pH for crystalline ribonuclease or fungus enzymes, as will be clear from the chapter on optimal conditions. But the method did not work when applied over a wider pH range.

Fungus enzyme material places its own limitations on the method as it is not optically indifferent to the same extent as crystalline ribonuclease. Spectrum measurements of the components of the reaction mixture in the ultraviolet range (Fig. 1)

showed that extinction of the enzyme controls at 300 mμ was due principally to the fungus. Hence the magnitude of the extinction values of blank solutions was determined by the concentration of the fungus enzymes in the reaction mixture. This must be taken into consideration when the spectrophotometric method is used to calculate the concentrations of the enzyme and the substrate in the reaction mixture. It was possible on the whole to obtain a fungus enzyme concentration in the reaction mixture which caused no absorption interference with the measurements.

Armillariella mellea and Lactarius torminosus species were chosen for further study of the preparation and purification of fungus ribonuclease. Although they do not represent the best species as far as activity is concerned, they were selected for the following reasons: (1) As the consumption of material was considerable the investigation object had to be a species of common occurrence; (2) Botanical identification was easy and there was no risk of error; (3) The colour from these fungi dissolved in the extracts was not particularly intensive; (4) Both had low phosphatase activity. The phosphatase activity of these species was found to be lower than that usually occurring in human sera (<0.4 Bodansky units). (5) Both showed small traces of proteolytic activity (30, 31); (6) DNase activity occurred to some extent only in Armillariella mellea (63).

The treatment of extracts of crude fungus powder was hampered greatly by the abundant oleaginous and resinous substances present in them. Chamberlain filtration, however, proved an effective method of removing them. After lyophilisation the yield was c. 10 per cent of the primary fungus material used for the extraction. The hygroscopic nature of the lyophilised enzyme preparations caused storage problems. It was possible, however, to overcome this difficulty by the semi-preparative paper electrophoretic method of further purification and repeated freeze drying. The humidity percentage was effectively reduced in this way and the nitrogen content remained roughly as before. At the same time, the colour diminished considerably. $(NH_4)_2SO_4$ fractionation was the last purification and preparation method. The nitrogen content of enzyme fractions thus prepared was nearly three times that of the primary material.

Enzyme activity was generally easy to extract from the crude fungus powder into distilled water. No great enzyme activity was found in the repeat extractions made from the indissoluble portion. No activity was found to remain in the filter paper after filtering. The strong colour of the extracts disturbed optical activity measurements, and the problem of colour elimination was not solved satisfactorily. Treatment with active carbon removed the colour completely, but left the filtrate inactive. It was not possible to eluate the enzyme back from active carbon. Treatment of filtrated fungus extract with lead acetate eliminated the colour to some extent without causing any noteworthy decrease in activity. Disappearance of colour also occurred in connection with paper electrophoretic purification of the enzyme. As the colour and activity of the preparations seemed to be associated in some way, and as the colour did not interfere decisively with the experiments, the matter was disregarded. In further purification by paper electrophoresis the activity of the lyophilisate remains unchanged or increases slightly.

Before undertaking the final $(NH_4)_2SO_4$ fractional precipitations for the improvement of enzyme activity, $(NH_4)_2SO_4$ titration was done from aqueous solutions of the lyophilisates of both the fungus enzymes. The object was to find suitable fraction limits. These limits were the following degrees of $(NH_4)_2SO_4$ saturation: Fraction I, 0–0.40; Fraction II, 0.40–0.60; Fraction III, 0.60–1.00. Subsequent precipitation of concentrated aqueous solutions of lyophilisates showed that the fractionation limits did not fully meet requirements. The fractionation of the Armillariella mellea preparation was an almost total failure and the yield was small. The Lactarius torminosus preparation precipitated best in a 0.40–0.60 saturation of $(NH_4)_2SO_4$ (Fraction II) and it was possible to separate as precipitate 77 per cent of the original activity without including the filtrate. The proportion of the precipitated fraction in the total yield was 95 per cent, of filtrate 5 per cent. It is true that the activity measurements of the filtrates do not give a true picture since a strong $(NH_4)_2SO_4$ concentration inhibits the reaction noticeably. Activity was generally found to diminish evenly when the degree of $(NH_4)_2SO_4$ saturation increased and at a saturation

of 0.9 the filtrate was completely inactive. The final results showed that $(NH_4)_2SO_4$ fractionation was an effective method of enhancing enzyme activity. Different fungus preparations behave in very different ways in salt precipitation, of course, and the best fractionation result was not always obtained from the primarily most active species. This was proved by the Lactarius torminosus preparation; the original activity of its lyophilisate was considerably smaller than that of the corresponding Armillariella mellea preparation but its $(NH_4)_2SO_4$ fractionation gave a much better and more reliable final result.

GENERAL DISCUSSION AND CONCLUSION

COMPARISON OF DIFFERENT RIBONUCLEASES

In comparison of the degrading effect on ribonucleic acid of the Armillariella mellea and Lactarius torminosus fungus enzymes with other ribonucleases, tobacco leaf ribonuclease (TLRNase) for instance may be taken in addition to crystalline pancreatic ribonuclease (PRNase) as the object of comparison. The special characteristics and mode of action of tobacco leaf ribonuclease have been studied throughly (43, 87). Equally detailed data are not yet available on the other ribonucleases.

The comparison was hampered by the absence of data on PRNase, TLRNase and fungus enzymes obtained in identical test conditions by parallel determinations. The methods employed were diversified and lacking in the detail required for their standardisation. Even small changes and differences in the reaction conditions cause divergences in the results. An important factor is the composition of the ribonucleic acid used as the substrate. The substrates were usually self-prepared, or at any rate self-purified, and hence their quality probably varied even considerably especially with a compound so labile as RNA. The impurities found in the substrate, e.g. metals and the spontaneous hydrolysis products of the substrate, might inhibit markedly the course of the reaction. Information is often lacking even on these important points. Finally, the degrees of purity of the above-mentioned enzymes differed considerably. This is naturally of great importance in the activity analyses. PRNase is a crystalline preparation free from impurities and side effects. The data on TLRNase were obtained from a specific preparation purified several times and with the side effects also eliminated. The fungus enzymes on the other hand must still be regarded

as crude material. Only the activity analyses were carried out with fractionated semi-purified preparations. These are the reasons precluding definitive comparison between these enzymes. Moreover, as the enzyme preparations in question differed considerably in specificity, detailed comparison could hardly be accurate. The following comparison therefore must be treated with reserve and understood as primarily indicative in nature.

The comparison, with the reservations mentioned, of *enzyme activities* is interesting. The specific activity of PRNase was determined in the present study as 46.5 Kunitz units per mg of dry substance. The specific activity of fungus enzyme (Lactarius torminosus, fraction II in $(NH_4)_2SO_4$ precipitation) advanced *ad* 1.4 Kunitz units per mg of dry matter. The activity of TLRNase, on the other hand, was expressed in arbitrary units (42) but was correlated with commercial crystalline PRNase. The unit of activity adopted was the quantity of TLRNase necessary to make 31 mg P/l of the substrate acid-soluble within an hour. This enzyme quantity corresponds to weak substrate solutions when 0.5 μg of PRNase was used. It could be calculated from this that the Lactarius torminosus fungus enzyme was c. 60 times stronger in activity in terms of Kunitz units than TLRNase. The Lactarius torminosus fungus enzyme, on the other hand, was c. 30 times weaker than PRNase expressed in the same units. These calculations should, however, be corroborated experimentally by parallel determinations in identical test conditions and especially on similar substrate.

Optimum pH was different for all the three enzymes. The best enzyme effect of PRNase covered a wide range: pH 7.0–8.2, optimum 7.7. The optimum pH of TLRNase was 5.5. The optimum pH has generally been 4.0 for fungus enzymes. Armillariella mellea had a second pH optimum at 7.1.

Thermostability was a feature common to all the enzymes. PRNase stands short-term boiling without losing its enzyme activity. Boiling for 30 min. between pH 2 and 5 caused an enzyme activity loss of only ca. 20 per cent. TLRNase is considerably more thermolabile but it, too, tolerates 3 min. of heating at +54 °C without loss of enzyme activity. At higher temperatures, however, its enzyme activity is destroyed. Of the fungus

enzymes, Armillariella mellea is relatively thermostable and retains c. 60 per cent of its enzyme activity after heating at +60 °C for 1 hour. The Lactarius torminosus fungus enzyme, on the other hand, is more heat-labile and is destroyed almost completely at the same temperature.

The enzymes differed in their *optimal temperatures*. The optimal temperature of PRNase is +65 °C at pH 5.0. There is no accurate information on the optimal temperature of TLRNase but after 4 hours' incubation at +37 °C and pH 5.0 its stability is unchanged and activity greater than with longer incubation at the same temperature or at +20 °C. Both fungus enzymes had the same optimal temperature at pH 4.0, viz. +40 °C. However, the optimal temperature of the Armillariella mellea fungus enzyme was +50 °C at its second pH optimum (7.1).

The *effect of various buffers* on enzyme activity was studied as the reaction mixture had to be buffered to prevent pH changes during the hydrolysis of RNA. The activities of PRNase and TLRNase were compared chiefly in citrate, maleate and picoline buffers or in their combinations, with the ion concentration ranging from 5 to 20 mM. The reaction velocity generally increased when the ion concentration of the buffer was raised. In this respect the citrate was the best of the buffers and its effect was the same either alone or in combination with other buffers. It may be inferred from this that the mode of action of citrate is based principally on the neutralisation of reaction-inhibiting factors rather than on the suspected inhibitory effect of other buffers. No comparable parallel determinations were made for fungus enzymes in the different buffers with either of the above-mentioned RNases. However, the effect of different buffers was studied at the same pH. Up to a concentration of 0.1 M no noteworthy differences were observed with acetate buffers of different strenghts and a Michaelis buffer series from which NaCl was omitted. On the other hand, the normal NaCl concentration in Michaelis buffer clearly inhibited the reaction.

It is difficult to draw conclusions of general applicability from the *inhibition studies*. Very significant differences may occur in enzyme activity when reactions of different calibrations or enzymes of different degrees of purity are used. Additional errors are caused by different substrate preparations. Even if

the same preparations were used, different concentrations caused errors as the reaction mixture contained different amounts of the metal cations occurring in the preparations as impurities. Moreover the inhibitory effect of these metal cations, e.g. Ca and Fe, is different in different pH ranges. It increases with the alkalinity of the reaction mixture. This is of importance, especially for PRNase the optimum of which lies on the alkaline side. Even though the same preparations and similar concentrations of these preparations at the same pH were employed, obvious errors were caused when the buffers differed. Before the results can be considered comparable all the sources of error mentioned above should be eliminated. This was not done in the present comparison.

It is a well-known fact that if, for instance, the concentrations of various metal cations are varied in the reaction mixture ordinary metals, inhibiting various enzymes, may also have an inhibitory effect on PRNase. TLRNase is even more easily affected in this respect. As for fungus enzymes degrading ribonucleic acid, the contrary is the case as regards the metal cations studied. Zn^{++} may be mentioned as a typical example. The activity of TLRNase decreases in maleate buffer to a third in the concentration of 16 μM Zn^{++} whereas 2.0 mM is needed to cause this inhibition with PRNase. The Lactarius torminosus fungus enzyme on the other hand is inhibited by the concentration of 2.0 mM Zn^{++} only slightly (Fig. 12) in Michaelis buffer from which NaCl has been omitted. Mn^{++} behaves in the same way.

The effect of *different substrates* on the digestive ability of enzymes was studied with PRNase and TLRNase. When RNA preparations made in different ways from yeast were employed as the substrate the enzyme effect depended on the inhibitory metal impurities in the substrate. These differences could not be established on digesting in citrate buffer. The greatest difference between the enzymes was that PRNase left an undigested "core" which could be precipitated with acids. With TLRNase no "core" formation was observable and the enzyme exhibited a greater affinity for RNA isolated from yeast than for RNA isolated from the tobacco mosaic virus. Nor was "core" formation established in the fungus enzymes when yeast RNA

was digested. The fungus enzymes resemble TLRNase in this respect.

On the other hand, when RNA isolated from the tobacco mosaic virus or tobacco leaf nucleoprotein was used as the substrate there was no notable difference in affinity between PRNase and TLRNase. Furthermore, the affinity of PRNase for these substrates was similar to its affinity for yeast RNA. Similar "core" formation was also observable with PRNase. The effect of the fungus enzymes on these substrates was not studied.

Using the "RNase resistant core" of yeast RNA as a substrate, PRNase naturally ceased to have any appreciable effect. TLRNase, on the other hand, disintegrated all "core" formations completely. Observations obtained from fungus enzymes with a yeast-RNA "core" point to the same direction.

Specificity differed greatly in all the enzymes. PRNase is a very specific phosphodiesterase which catalyses the cleavage only of certain strictly defined internucleotide bonds, viz. those between the 3'-pyrimidine nucleoside phosphoryl groups and the 5'-hydroxyl groups of adjacent purine or pyrimidine nucleotide groups. The products of enzymic hydrolysis consist of pyrimidine mononucleotides, representing a large portion of the pyrimidine content of the RNA, easily diffusible di- and trinucleotides and a ribonuclease-resistant nucleic acid residue or "limit polynucleotide" forming what has been termed the "core" of the RNA molecule. Thus the main action of ribonuclease on RNA is the splitting off of pyrimidine mononucleotides (57, 93).

The mode of action of purified TLRNase on RNA is quite different. Unlike the PRNase, this enzyme cleaves all the diester bonds and gives rise to purine and pyrimidine cyclic nucleotides. While the pyrimidine cyclic nucleotides are inert to further action of this enzyme, the purine cyclic nucleotides are slowly hydrolysed to nucleoside 3'-phosphates (87).

Of the fungus enzymes, the mode of action of Lactarius torminosus lyophilisate alone was examined. The preparation was not purified selectively in any way. It appeared in fact that there was probably not only one enzyme but several. Judging by the degradation products, the effect of Lactarius

torminosus took place in two phases. Firstly, RNA was depolymerised very rapidly into acid-soluble degradation products, probably to the nucleotide stage, which disintegrated immediately into nucleosides under the influence of the phosphatase contained in the fungus enzyme, whereupon abundant inorganic phosphorus was liberated. This view was substantiated by the time difference between the conversion of the substrate to acid-soluble form and the starting time of the liberation of inorganic phosphorus. However, it was not possible to isolate nucleotide as intermediate products from the reaction mixture. This would seem to indicate a relatively pronounced phosphatase effect. Further studies to clarify this point are in progress (64). The experiments performed so far indicate that Lactarius torminosus fungus enzyme lyophilisate liberates the same number of mols of inorganic phosphorus from each purine and pyrimidine 5'-ribonucleotide in the same reaction time. However, not all the nucleotide phosphorus was converted to inorganic form within a reaction time of 90 hours. When different sugar phosphates were used as the substrate, on the other hand, the ribose specificity of the Lactarius torminosus fungus enzyme was obvious. Within a reaction time of 35 hours practically all the phosphorus of ribose-5-phosphate (Barium salt, Sigma Chemical Company) was converted to inorganic form. On the other hand, only 30 per cent of the phosphorus content of glucose-6-phosphate (Barium salt, Sigma Chemical Company) and fructose-1,6-phosphate (Sodium salt, Sigma Chemical Company) was converted to inorganic form within the same reaction time.

Fig. 40 gives a summary comparison of the hydrolysis by chemical methods of ribonucleic acid and PRNase and the Lactarius torminosus fungus enzyme. The acid hydrolysis was performed by boiling ribonucleic acid at +100 °C for 1 hour with 1 N HCl and neutralising the hydrolysate with 2 N NaOH. Alkaline hydrolysis was performed with 1 N NaOH at room temperature for 24 hours. Using crystalline pancreatic ribonuclease, ribonucleic acid was hydrolysed for 3 hours at +37 °C in a reaction mixture in which the substrate concentration was 0.5 per cent and the enzyme concentration 0.0001 per cent, and pH 7.8. The ribonucleic acid was hydrolysed with fungus enzyme lyophilisate as shown in Fig. 20.

The acid hydrolysate was analysed with tert.-butanol—hydrochloric acid—water solvent. The running time was 45 hours, at +20 °C. The other hydrolysates were analysed with 0.1 M phosphate buffer pH 6.8 — ammonium sulphate — n-propanol solvent. The running times were 10 hours, at +20 °C.

All the hydrolysates were also analysed simultaneously (Fig. 41) and in the same conditions with n-butanol — water solvent. The running time was 40 hours, temperature +20 °C.

The solvents, hydrolysate concentrations at the starting points and the running times were deliberately chosen empirically to give uniform results from the different hydrolysates. The aim was to demonstrate only type chromatograms obtainable by means of different hydrolysis methods. No accurate comparison of the different hydrolysis methods is possible from these chromatograms.

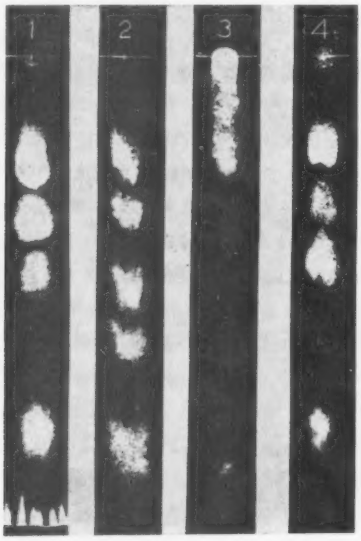

Fig. 40. — Chromatographic illustration of the various ribonucleic acid degradation methods.

Lane 1. — Hydrochloric acid hydrolysis of RNA. Solvent: tert.-butanol—constant boiling hydrochloric acid—water, 70/13/17, 45 hours, at +20 °C with the ascending technique. Spot: 20 μl of 1 per cent neutralised hydrolysate of RNA.

Lane 2. — Alkaline hydrolysis of RNA.

Lane 3. — PRNase hydrolysis of RNA.

Lane 4. — Degradation of RNA with Lactarius torminosus fungus enzyme. Solvent (Lanes 2, 3 and 4): 0.1 M phosphate buffer pH 6.8— ammonium sulphate — n-propanol. 100/60/2, 10 hours, at +20 °C with the ascending technique. Spots: c. 20 μl of 1 per cent hydrolysate of RNA.

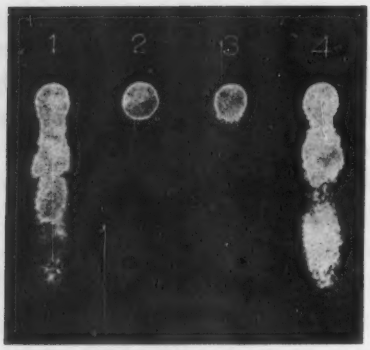

Fig. 41. — Chromatographic illustration of the various ribonucleic acid degradation methods.
Lane 1. — Hydrochloric acid hydrolysis of RNA.
Lane 2. — Alkaline hydrolysis of RNA.
Lane 3. — PRNase hydrolysis of RNA.
Lane 4. — Degradation of RNA with Lactarius torminosus fungus enzyme.
All the hydrolysates were analysed by the same solvent: n-butanol — water, 86/14, 36 hours, at +20 °C with the ascending technique. Spots: c. 20 μl of 1 per cent hydrolysate of RNA.

SUMMARY

The ability of ordinary fungi to degrade ribonucleic acid was the object of the present work. The enzymatic properties of two fungi possessing such enzyme activity were also determined for the charting of optimal conditions of function. Further, the degradation products formed from ribonucleic acid under the effect of such fungus enzyme were identified as far as possible. Finally, enzyme activity was enhanced by purifying two crude fungus enzyme preparations.

The investigation material consisted of 242 fungus species divided into three groups: *Myxomycetes* 2, *Ascomycetes* 8 and *Basidiomycetes* 232 species. 211 of these fungi, i.e. 87 per cent, showed a distinct enzyme activity degrading ribonucleic acid. Enzyme activity was very pronounced in 83 fungi, i.e. 34 per cent of the total material. The ribonucleic acid degrading effect of the fungus enzymes did not appear to follow the systematic order of the fungi. It was established in very broad outline that the fungi known to be mycorrhizal had a more pronounced degrading effect on ribonucleic acid than decaying fungi, especially fungi decaying wood. Exceptions to this, however, were observed.

The very active Armillariella mellea and Lactarius torminosus fungi were chosen for the further studies concerned with the special properties of fungus enzymes. The pH optimum was 4.0 in both the species, but Armillariella mellea also had another pH optimum, 7.1. The fungi differed in thermostability. Armillariella mellea was relatively heat-stable, tolerated heating at +60 °C for 1 hour to the extent that it retained c. 60 per cent of its original activity. Lactarius torminosus proved more thermolabile, was destroyed practically in its entirety at +60 °C. The optimal temperature at pH 4.0 was +40 °C for both the fungus enzymes. The optimal temperature of Armillariella mellea at pH 7.1 was +50 °C. The reaction velocity was greater

with Armillariella mellea at the acid pH optimum. The effect of the different concentrations of the enzyme and the substrate on the reaction rate with Armillariella mellea, at both pH optima and +37 °C, concurred with the theory of Michaelis-Menten. The inhibitory effect of different metal cations was examined on Lactarius torminosus fungus enzyme. Cu^{++} was found to have the greatest inhibitory effect, though in a relatively high concentration. No specific inhibitor was found among the substances studied, nor did any of them activate the enzyme.

For analysis and identification of the degradation products, ribonucleic acid was digested by the Lactarius torminosus fungus enzyme. The degradation products were all acid-soluble. No "core" formation resistant to fungus enzyme was observed. The fungus enzyme rapidly split the ribonucleic acid to degradation products smaller than the nucleotide degree as generous quantities of inorganic phosphorus were liberated and the reaction end products admitted of paper chromatographic isolation with the aid of several solvent systems. Using known ribonucleic acid derivatives as controls, the reaction end products were identified from the rf-values as ribonucleosides. The ribonucleoside character of the degradation products eluated from chromatography paper was further confirmed by determining their specific ultraviolet spectra and absorbancy ratios spectrophotometrically, and by phosphorus (P^{32}) determinations. No ribonucleotides were found among the reaction end products. Finally, the quantitative isolation of ribonucleosides was performed. 45.3 per cent of the ribonucleic acid was degraded to ribonucleosides during 24 hours of Lactarius torminosus fungus enzyme digestion. The following quantities as percentage molar proportions of different ribonucleosides were liberated: adenosine 32.5, guanosine 35.7, uridine 12.7 and cytidine 19.0. The amount of 6-keto compounds and 6-amino compounds liberated was thus very much the same.

The mode of action of the Lactarius torminosus fungus enzyme was assessed from the degradation products. With impure Lactarius torminosus, which probably contains several different enzymes, ribonucleic acid was degraded first by fungus enzyme depolymerase to acid-soluble mono- or oligonucleotides which immediately disintegrated further under the influence of the

phosphatase contained in the fungus enzyme to the nucleoside degree and liberated inorganic phosphorus. Hence it should be impossible for nucleotides to originate and this explained why they were not established in the reaction mixture in any phase of the enzyme digestion by the method used.

To enhance enzyme activity, Armillariella mellea and Lactarius torminosus fungus enzyme lyophilisates were purified by $(NH_4)_2SO_4$ fractional precipitations. The effectiveness of the purification method was followed continuously in the different fractionation phases by comparing the activities of the intermediate products with one another and with crystalline pancreatic ribonuclease in terms of Kunitz units. This was possible because the fungus enzymes, in the same way as crystalline pancreatic ribonuclease, caused a decrease in the extinction of ribonucleic acid at pH 5.0 and 300 mμ. Armillariella mellea did not give a satisfactory fractionation. On the other hand, it proved possible by $(NH_4)_2SO_4$ saturation to isolate from Lactarius torminosus lyophilisate, with an original activity c. 10000 times weaker than that of crystalline pancreatic ribonuclease, a precipitate fraction between 0.40-0.60 containing 77 per cent of the original activity and, calculated in Kunitz units per mg of dry matter, only some 30 times weaker than crystalline pancreatic ribonuclease. The increase in activity was thus c. 330 times that of the primary material.

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